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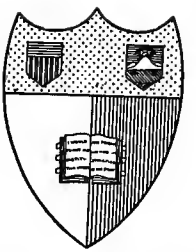

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THE BACTERIOLOGICAL EXAMINATION OF WATER-SUPPLIES

THE

BACTERIOLOGICAL EXAMINATION OF WATER-SUPPLIES

BY

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DEDICATED

TO

DR. A. C. HOUSTON,

Director of Water Examinations, Metropolitan Water Board,

IN ADMIRATION OF HIS PROLONGED AND LABORIOUS WORK UPON THE PROBLEMS WHICH UNDERLIE THE BACTERIOLOGICAL EXAMINATION OF WATER-SUPPLIES; WORK WHICH HAS DONE MUCH TO REMOVE THE SUBJECT FROM THE REGIONS OF CONJECTURE AND ASSUMPTION TO THOSE OF DEFINITELY ASCERTAINED FACT.

PREFACE

THE writing of a book upon the bacteriological examination of water presents numerous difficulties. Many of the facts upon which such an examination rests have been so recently ascertained, that their value is not readily to be appraised, and difficulty is experienced in selecting those essential and important.

My intention has been to demonstrate the facts which are now established, and to bring the varied data into some sort of ordered relationship.

Dogmatic opinion has been avoided, the subject being considered from a critical standpoint, and practical conclusions drawn only when they seem justified by the available evidence.

Unfortunately, many of the data upon which the bacteriological examination of water is based have not passed beyond the regions of controversy, and on not a few questions—some of which are of much importance—each bacteriologist is, at present, a law unto himself. For certain parts of the subject it has, therefore, not been possible to present any one view as the united and accepted opinion of those whose knowledge entitles them to sit in judgment.

In such cases I have not hesitated to give the available

facts, and from them to draw definite conclusions for the guidance of those inexperienced in this difficult subject, being emboldened to such a course by an extensive experience in the bacteriological examination of water, extending over the past seven years.

This work has been associated with continuous research upon soil, excreta, etc., carried out with the special object of elucidating some of the more obscure problems vitally associated with the bacteriology of water. The results of many of these investigations have been previously published in the various scientific journals, but some of them are here referred to for the first time.

With regard to one very controversial matter, I have tried to deal fairly with the question of the significance of aberrant coli-like organisms. This troublesome problem is usually dealt with by drawing attention to it as a difficulty, and then either leaving it unanswered by saying that each worker has his own opinions, or by an unsupported dogmatic statement of the views of the particular writer. The significance of the presence of these organisms is one which *must* be considered in actual practice.

The bacteriological examination of water has passed through several phases. At first it was hailed as a direct, certain method by which to test the purity of a water; then it lost credit as limitations to its use (based on insufficient knowledge) were met with. Now, owing to the prolonged labours of many observers (largely English and American), it is taking its rightful place as the most valuable of all available methods by which to judge the purity of a water-supply.

To-day it may be said that the bacteriological examination of water has been established on a scientific basis of ascertained fact. This is not to deny that there are many difficulties which only patient investigation can clear up, but our knowledge is established along the broad fundamental lines of the subject, and these difficulties are more or less subsidiary.

This being my opinion, I think the present a suitable time for a book dealing, not merely with the details of practical procedure, but also with the data upon which these methods are based, and with the deductions held to be justifiable from them.

Several of the illustrations are taken from the bacteriological sections contributed by the writer to Kenwood's 'Public Health Laboratory Work.'

I am indebted to Messrs. J. and A. Churchill, Charles Griffin and Co., and W. B. Saunders and Co., for permission to use illustrations, and to Messrs. Baird and Tatlock for the loan of blocks; also to the Cambridge University Press for permission to reprint the table from the *Journal of Hygiene*.

I have to thank Major Freeman, R.A.M.C., for useful suggestions, while in particular I would acknowledge my indebtedness to Dr. Penry Rowland for his valuable assistance in reading and revising the proof-sheets.

W. G. Savage.

September, 1906.

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THE

BACTERIOLOGICAL EXAMINATION OF WATER-SUPPLIES

CHAPTER I

Influences affecting Bacteria in Water

Bacteria are almost universally distributed, but the numbers present and the kinds existing are not fortuitous, but subject to definite conditions of food-supply, moisture, and the like. All recent bacteriological work is in the direction of showing conclusively that each species of organism has both its natural habitat and its accidental loci.

In the former, bacteria live and flourish, although governed by definite influences which limit their multiplication; in the latter they tend to die out.

Certain of these influencing factors seem to be common to all organisms, others are peculiar to special groups.

Each variety has its own suitable conditions of environment, and if these are not satisfied the organism will ultimately die out, although it may be able to resist these adverse conditions for considerable periods.

It follows from this that water, soil, etc., tend to have their own bacterial flora, and although the bacteria entering are many and varied, yet, if the influences which make up the environment are given time to exert themselves, certain

types and varieties will predominate, while others will be eliminated.

In the case of fluids such as sewage, there is scarcely time for these factors to come into efficient play, but for many water-supplies such influences do exert themselves powerfully, and it is not without meaning to speak of a bacterial flora natural to water. Such a term implies that in water there are organisms present, which find the conditions they require to enable them to multiply and survive for almost indefinite periods. The number of even these more or less naturally present bacteria varies greatly with different kinds of waters and with varying circumstances.

Under natural conditions, adventitious substances containing bacteria are constantly being added to water. For example, a pure mountain stream is constantly, in times of heavy rain, having added to it washings from the soil. These washings contain bacteria, such as *Bacillus mycoides* and *Bacillus mesentericus*, abundant in soil, but which do not readily flourish in water, and which there, after a more or less precarious existence, die out.

Yet, again, the adventitious substances added may not be unobjectionable. They may be, in part, composed of washings from recently manured soils, or contain sewage more or less purified. For the organisms from such sources, also, the conditions under which they find themselves in water are more or less unsuitable, and, given sufficient time, most if not all of them will be eliminated.

The bacteria found in water naturally group themselves into three classes:

1. The more or less natural water bacteria—organisms which are widely distributed and present in most waters, and which in water find a suitable environment; for example, *B. fluorescens liquefaciens. Normal inhabitants.*

2. The bacteria added to water from unobjectionable sources, such as pure soil washings; for example, *B. mycoides. Unobjectionable aliens.*

3. The bacteria gaining access to water from polluted sources—*e.g.*, sewage or animal excreta; for example, *B. coli communis*. *Objectionable aliens.*

That purification, and alteration of flora to a condition of more or less equilibrium, goes on has been abundantly shown by the work of a great number of observers. Thus, laboratory experiments strongly favour the view that typhoid bacilli introduced into any water-supply rapidly die out. Sewage entering a river will charge the water with vast numbers of its own bacteria alien to a pure water, yet twenty or thirty miles lower down, if no fresh additions have been made between, the water will be free, or practically free, from these organisms of sewage origin.

The conditions which make for such purification are worthy of attention.

Mechanical agitation has been shown by Miquel, Cramer, Tiemann and Gärtner, Scheurlen, and others, to have no influence upon the number of organisms in a water.

The influence of **light**, and especially of **sunlight**, upon bacteria has been recognised since the classical researches of Downes and Blunt in 1877. How far sunlight is a practical means of purification of streams and natural waters generally is still doubtful, in spite of extended investigations. Buchner, in clear water, traced some degree of bactericidal influence to a depth of 2 metres, and very slight influence to a greater depth. The intensity of the light and the quantity of suspended particles in the water are obviously powerfully influencing factors. Clark and Gage (Clark and Gage,* 1902) recently tested the action of sunlight upon typhoid bacilli in water, and found that when this organism was mixed with water and placed in Petri dishes in the sun an exposure of about four hours was necessary to kill all the typhoid bacilli, although the

* The names in brackets refer to the Bibliography, where the references are given in full.

numbers were markedly reduced after a fifteen minutes' exposure. Very similar results were obtained with *B. coli*. As a practical agent in the purification of streams, even when these are shallow, the influence of light is probably not of great importance.

The influence of **temperature** is of some importance. In warm weather the conditions are more favourable to rapid multiplication, but under natural conditions this factor is always associated with others, such as the amount of dilution, presence of surface washings, etc., and not infrequently fewer organisms are met with in summer.

Thus, for the years 1886, 1887, and 1888 Frankland obtained the following results with water from the Thames at Hampton. The figures are the numbers of bacteria per c.c. developing upon gelatine plates.

Month.	1886.	1887.	1888.
January	45,000	30,800	92,000
February	15,800	6,700	40,000
March	11,410	30,900	66,000
April	12,250	52,100	13,000
May	4,800	2,100	1,900
June	8,300	2,200	3,500
July	3,000	2,500	1,070
August...	6,100	7,200	3,000
September	8,400	16,700	1,740
October	8,600	6,700	1,130
November	56,000	81,000	11,700
December	63,000	19,000	10,600

Here the summer months show the lowest number of bacteria, this being due to the fact that during dry weather the source of the water is mainly from springs, while in the winter the washings of much cultivated land are received.

The **chemical composition of the water**, except under exceptional circumstances, does not play a part of any importance as far as the ordinary aërobic bacteria, which develop upon gelatine media, are concerned.

The **quantity of food material** present is of great importance. Jordan (Jordan, 1900), in discussing the self-purification of the Illinois River, remarks that 'the decomposition of large quantities of albuminous substance is first accompanied by great bacterial reproduction, and that is invariably followed by a season of speedy and extreme mortality of the bacteria. In the causes connected with the insufficiency or unsuitability of the food-supply is to be found, I believe, the main reasons for the bacterial self-purification of streams.'

In their studies upon the self-purification of the river Severn at Shrewsbury, Boyce and his co-workers (Boyce, MacConkey, Grünbaum, and Hill, 1902) found that the bacterial purification was parallel to a diminution of organic matter. As they remark (p. 107), 'without food there is no multiplication, and if there is no multiplication, the bacteria, like other particles in suspension, undergo gradual sedimentation, and adhere to the sides and bottom of the river.' These observers attached considerable importance to protozoa and higher forms of animal life, and also to algæ and river plants, as aids in the destruction of organic matter and self-purification of the river.

The following table from their report illustrates the diminution in the number of organisms, due to self-purification, for several rivers :

RESULTS OF BACTERIOLOGICAL EXAMINATION OF VARIOUS RIVERS AT AND BELOW LARGE SOURCES OF POLLUTION.

	Cologne : River Rhine.		Munich : River Isar.	Zürich : River Limmat.	Grützow : River Warnow.	Dortmund : River Emscher.	Berlin : River Spree.	
Above	4,786	2,000	305	1667	1,810	—	Above... ...	8,951
About 0·6 miles below	—	—	9,387	13,336	94,500	1,453,000	Just below ...	243,587
,, 2·7 ,,	—	—	13,503	6,045	—	—	3·5 km. below	343,332
,, 6·0 ,,	30,432	9,010	8,764	3,263	12,970	124,000	11·0 ,,	170,143
,, 12·0 ,,	12,460	3,690	4,796	—	—	220,600	14·0 ,,	130,700
,, 15·0 ,,	9,595	6,830	3,602	—	—	—	16·5 ,,	175,048
,, 26·0 ,,	7,869	4,720	—	—	850	—	—	9,190

The **inhibiting action of many microbes and their products** upon one another is well known, and will be referred to subsequently from another point of view. This action may have some slight influence upon the purification of water-supplies.

Of all the factors at work, however, that of **sedimentation** is undoubtedly the most potent.

Bacteria have weight, and therefore obey the law of gravitation. Sedimentation acts in two ways, causing not only a subsidence of the actual bacteria, but also a sinking of the organic particles in the water. This action, besides carrying down mechanically vast numbers of bacteria, deprives the water of its nutritive properties, rendering it less suitable as a source of food-supply for the organisms yet remaining in it.

The influence of sedimentation is most clearly shown when large masses of water are stored in reservoirs, but it is also exerted in rivers and other bodies of water.

A number of instances of reduction of micro-organisms by storage are recorded in the Franklands' valuable work on 'Micro-organisms in Water' (G. and P. Frankland, 1894). As a typical example, P. Frankland's examination of the West Middlesex Company's water may be cited (p. 188). Samples were collected of the Thames water at Hampton, of the same water after having passed through one storage reservoir only, and of the water after passing through two storage reservoirs. At Hampton the number per c.c. was 1,437, after passage through one storage reservoir 318, and after passage through two storage reservoirs 177.

Jordan, in the paper already quoted from, found that during a flow of twenty-four miles the Illinois River became nearly free from the great mass of sewage bacteria with which it was originally laden, the bacterial content of the river twenty-four miles below the great source of pollution being not greatly in excess of that of the local tributary streams. Discussing the causes of their disappearance, he dismisses

mechanical agitation and aeration as having no influence, while for this river, dilution and sunlight can be neglected as influencing factors. As regards the action of sedimentation, and dealing with this particular river, he remarks, 'There can be no doubt that the various influences summed up by the word "sedimentation" are sufficiently powerful to obviate the necessity for summoning another cause.' As already mentioned, sedimentation and food-supply are closely related, so these remarks are not in contradistinction to those already quoted.

The purification of rivers and natural waters generally is a complex matter, and many factors doubtless contribute, the relative importance of which varies with the different waters; but it cannot be doubted that the three essential factors at work are the influence of sedimentation, the diminution of food-supply, and the mechanical influence of dilution with purer waters. In regard to these factors, it must be remembered that they do not make for a uniform reduction of all the organisms, but that a certain amount of selective action is exerted. The more delicate and alien bacteria are reduced in greater proportion than those naturally present in water.

As Delépine (Delépine, 1901) has pointed out, the self-purification of rivers is not a phenomenon peculiar to open streams. He has shown that unfiltered water carried through mains undergoes a considerable amount of bacterial purification during its transit, so long as the normal flow is not modified by violent flushings or other disturbances. In investigating the influence of **pressure** upon self-purification, Delépine, examining the Manchester water at different levels, found no relation whatever between pressure and the number of bacteria.

Returning to a consideration of the classes of bacteria found in natural waters, it will be at once recognised that it is not merely, or primarily, the number of bacteria, but their varieties and source, which is of moment.

The organisms of Class I. (normal inhabitants) may be found in large numbers, but if only this class is present contamination is not indicated. Their presence is not, however, devoid of all significance, since the number of organisms is most influenced by the quantity of foodstuff available in the water, and, other things being equal, their presence in large numbers is a measure of increased organic matter, but not necessarily of harmful organic matter.

The organisms of Class II. (unobjectionable aliens) give indications of conditions not in themselves prejudicial, but which are liable, under altered circumstances, to become objectionable. Pure soil washings are not, as such, in any way prejudicial, but the soil washings may not be always unobjectionable, and the presence of organisms of this class is an indication of possible future danger. Thus the addition of flood washings of cultivated soil to water is distinctly objectionable. In such a case Class II. organisms would be accompanied by those of Class III.

Thresh (Thresh, 1903), in August, 1903, described an epidemic of diarrhœa due to the use of impure water. There were over 1,000 cases and 14 deaths. The outbreak was due to heavy rains sweeping washings from the adjacent soil into a small reservoir to which the public water gained access. The soil around consisted of garden ground manured from time to time with road sweepings. The water-supply itself, apart from this local pollution, was an uncontaminated one.

The bacteria of Class III. (objectionable aliens) are directly indicative of danger, and, other things being equal, the greater their number, the more immediate and massive the dangerous contamination.

If we had means by which we could separately estimate the numbers of each class in a given sample of water, the value of such an estimation could not be exaggerated, but the distinctions between these three groups are, in practice, not sharply defined. By picking out, however, the more

common and typical representatives of these classes, and estimating their numerical presence, we are enabled to gauge the relative proportions of these groups in any given sample.

At one time the conditions which influence the presence and distribution of bacteria in water were not clearly understood, and arbitrary standards of the number of organisms permissible in water-supplies were set up by Miquel, Macé, and other workers, and these supplies were classed as very good, good, bad, very bad, etc., according to the number of bacteria developing upon gelatine plates.

But the varying factors at work make it obvious that each class of water must be considered separately, and that even if it were possible to devise standards applicable to one class, they would be wholly inapplicable to others. For example, deep well water is water which, whether initially pure or impure, has filtered through a considerable depth of soil. Soil in its deeper parts is germ-free, while it also acts as a most efficient filter; it adds nothing to, but filters out the bacteria from the water, so that, theoretically, a deep well water should be quite free from micro-organisms. Inasmuch as surface water from the mouth frequently obtains admission to a limited degree, or slight flaws may be present in the superimposed strata, organisms in small numbers do gain access, and, if the conditions are favourable, may flourish. In pure protected deep well waters the number of organisms should be all of Class I., and very few in number.

The presence of Class III. organisms in such a supply, even in quite small numbers, would be sufficient to prejudice the bacteriologist strongly against it.

On the other hand, a water derived from pure upland surfaces will contain not only a considerable number of Class I. organisms, but also bacteria derived from the washings of the soil—all organisms, it may be, of no harmful import. The number of organisms will be considerably

greater than is found in deep well water, and yet both may be equally free from any dangerous pollution.

Standards of number or of kind, applicable to the one class of water, are totally inapplicable when applied to the other class.

Practical experience has shown that the number of organisms in a pure water remains reasonably constant, or at least that there are but seldom violent fluctuations. The conditions which make for multiplication and increase are met by those which make for elimination and decrease, and a more or less stable balance results. For the *routine* examination of the same water simple enumerations are then undoubtedly of value.

Used alone, such enumerations do not differentiate between the probable sources of the different organisms. For individual samples the difficulties of a correct interpretation of a numerical increase or decrease are so great that simple enumeration is insufficient to enable an opinion to be given as to the purity of any given supply. It is to the special determination of organisms indicative of objectionable pollution that we have to look for accurate diagnostic data.

Here, again, difficulties arise. We cannot estimate and differentiate with our present knowledge all the bacteria gaining access to water from objectionable sources.

In default of this, bacteriologists have with great care ascertained the bacteria most numerous in such sources, and have devised means to enumerate these, and from their presence and numbers to detect the existence and deduce the amount of such objectionable pollution. Of these organisms of indication, *B. coli* and allied organisms, streptococci and *B. enteritidis sporogenes*, have the greatest claim to consideration.

The basis on which this claim is made, the characters and methods of detection of these organisms, and the deductions possible from their enumeration, will be considered in detail in subsequent chapters.

CHAPTER II

The Quantitative Bacterial Content of Natural Waters

A CONSIDERATION of Chapter I., which shows the factors at work influencing bacteria in water, will make it clear that a determination of the actual number of organisms in water-supplies is of less value than was originally supposed; but since it is of considerable importance in certain cases, and of some value in all, further consideration is necessary.

The ordinary method of enumeration consists in adding definite, but varied, quantities of the water under examination to melted tubes of nutrient gelatine, and frequently of nutrient agar also, mixing thoroughly while still liquid, pouring out into Petri dishes, and allowing to solidify. The 'plates' or Petri dishes are then incubated at 20° to 22° C. (or sometimes at room temperature) for the gelatine, and at 37° C. for the agar media.

The colonies which develop are counted after appropriate periods of time, and from this the number of organisms in the original water is calculated.

In principle the method is admirable, and in practice it is not difficult or burdensome to carry out; but, from the point of view of scientific accuracy and reliability, it is liable to many fallacies and subject to many limitations.

In the first place, the figures obtained do not, and cannot, give the total number of organisms present in the water examined. It is well known, for example, that the very

abundant nitrifying organisms in water will not develop on such plates, nor will the organisms which are strict anaërobes grow under these conditions. Certain bacteria will develop only at temperatures above or below those used for incubation.

In other words, the figures obtained by such enumerations only mean that, under the conditions of the experiment, a certain number of organisms in a definite quantity of water were able to propagate and multiply.

This method of enumeration also assumes that during the mixing all the organisms will become separated one from another, that in the plate they will become fixed apart from each other, and that therefore each developing colony indicates one organism, and only one, in the original quantity of water used. This is a near enough approximation for practical purposes, but it is certainly not altogether accurate.

A much more important limitation than any of the above is the well-ascertained fact that the number of colonies which will develop varies markedly with such highly technical matters as the exact composition of the medium, its chemical reaction, and the time and temperature of incubation.

Ordinary nutrient gelatine and agar are not chemically simple, but very complex bodies. The beef extract which forms their foundation is very variable. In many laboratories it is made from fresh beef, while in others the various meat extracts on the market are employed. The composition of both the fresh beef infusions and the commercial extracts are variable. Gage and Adams (Gage and Adams, 1904), for example, showed marked variations in the composition of beef infusions. After the albumins were removed by coagulation and filtration, they found a variation of nearly 1 per cent. in the organic solids, and a variation in the reaction of the infusion of 1·3 per cent. As they point out, we are thus using as a basis for a

nutrient medium, supposed to be of constant composition and reaction, a substance the natural variations of which are greater than the total amount of accurately determined nutrients (1 per cent. peptone) which we incorporate with it.

The infusions made from beef extracts, while probably somewhat less nutritive for highly pathogenic organisms, are of more uniform composition, and sufficiently nutritive for the ordinary purposes of water bacteriology. Different brands of commercial extracts yield, however, differing results.

Sedgwick and Prescott (Sedgwick and Prescott, 1895) found that the percentage of peptone is important, while the source of the gelatine also influences the result, hide gelatine giving the highest figures. Not only the amount but also the variety of peptone used influences the number of organisms developing. Thus Gage and Adams, in the paper just quoted, obtained, as a rule, higher results with Witte's peptone than with Merck's peptone, the other ingredients being the same.

Recent American workers (*e.g.*, Gage and Phelps, 1902, *a*) have pointed out that the omission of common salt gives higher figures, and the American Committee on Standard Methods of Water Analysis (Report of Committee, 1905) recommends that sodium chloride be not added to any of the specific culture media.

Of even more importance than the precise composition of the medium is its chemical reaction. The influence of varying reaction upon the number of developing colonies has been recognised for a good many years, although even at the present time, in many laboratories, but little attention is paid to this important factor.

Behring in 1888 estimated the reaction of his media by titration methods, using rosolic acid as the indicator.

Schultz (Schultz, 1891) pointed out the importance of the reaction of the medium, and that for this estimation litmus-paper was not sufficiently accurate. After extensive investigation with different indicators, he adopted phenolphthalein as

the most suitable. In his method 1 c.c. of the culture medium is removed to a test-tube, a drop of phenolphthalein solution added, and 0·4 per cent. caustic soda added drop by drop until a faint pink colour appears. Three separate titrations are made, and, from the mean result, the total alkali required to neutralize the rest of the medium is calculated, and this quantity is added as 4 per cent. caustic soda.

Reinsch (Reinsch, 1891) carefully studied the influence of varying amounts of sodium carbonate upon the number of bacteria in water which would develop upon peptone-gelatine plates. He used contaminated water from the river Elbe below Hamburg. He found that the greatest number of colonies was obtained when 0·1 per cent. of sodium carbonate had been added to the already slightly alkaline gelatine medium. On the other hand, the addition of tartaric acid caused a diminution in numbers, the highest figures being obtained by using the faintly alkaline medium without the addition of any acid, each addition of acid causing a diminution in numbers. Reinsch also drew attention to the unreliability and variability of litmus-paper, and recommended accurate titration as described by Schultz.

Since 1891 a number of investigations have been made upon the chemical reaction best suited for enumeration work. Of these investigations those made in connection with the American Committee on Standard Methods of Water Analysis, those of Eyre in this country, and of Hesse in Germany, are of most importance.

The American Committee (1905) recommends (p. 82) that 'for general work the standard reaction shall be + 1 per cent., but for long-continued work upon water from the same source the optimum reaction shall be ascertained by experiment and thereafter adhered to.'

Eyre (Eyre, 1900 and 1901) showed the importance of selecting the right indicator, and discussed the causes making for want of uniformity of reaction. He suggested the use of media having a reaction of + 10—*i.e.*, a medium every litre

of which would still require the addition of 10 c.c. of *normal* caustic soda to render it neutral to phenolphthalein.

This standard was adopted and recommended for use by the English Committee on Standardization of Methods for the Bacterioscopic Examination of Water (Report of Committee, 1904).

+ 10 on Eyre's scale is the same as + 1 per cent., so the English and American Committees are in close accord upon this point, the only difference being that in the titration Eyre recommends stopping the alkali addition at the first sign of a pink colour, while the directions of the American Committee are to add until a 'faint but distinct pink' appears.

To obtain media of definite reaction is by no means a simple matter, and there are many possibilities of error. Thus Fremlin (Fremlin, 1901-02) has pointed out that the reaction of media alters with the time since preparation, media losing slowly, steadily, and progressively the faintly alkaline reaction that they possess when freshly prepared.

Gustav Hesse (Hesse, 1904) showed that during sterilization an alkaline agar solution loses a not unimportant quantity of alkali, and this even with a simple agar and water solution. He further showed that there is a slight loss even on merely boiling, while the greater the heating, the greater the loss of alkalinity. Experiments with gelatine and with bouillon showed a similar decrease of alkalinity.

He also demonstrated that the kind of glass of which the flasks used for the preparation of media were composed exerts a considerable influence upon the reaction of the contained fluid. The different kinds of glass experimented with—Jena, Berlin, Bohemian, etc.—allowed different quantities of alkali to be dissolved out. When Nährstoff-Heyden agar (for composition see p. 22) was made and sterilized in different kinds of glass vessels, and this agar then used for estimating the bacteria in tap water, markedly differing numbers of colonies were obtained on the different plates, although each contained the same amount of the water.

Hesse recommended that in the sterilization of media, glass which does not give up alkali should alone be used. In his experiments Jena glass was found to be practically unacted upon.

Even with media of, as far as possible, identical composition and standard reaction, differing results will be obtained, if differing temperatures of incubation and times of incubation are employed. With gelatine plates the period of incubation allowed in different laboratories is very variable, some workers counting after two days, others after three days, others daily, until liquefaction prevents further enumeration, or until the number of colonies remains constant.

The method of counting is variable, some counting all the colonies which can be made out with the naked eye only, others by using a hand lens, others a low power of the microscope.

The temperature of incubation for gelatine plates is, generally, 20° to 22° C., but some workers still incubate at laboratory temperatures.

A difference of practice in these matters will obviously result in the obtaining of very different figures.

These considerations, now well known, show clearly how readily very different results may be obtained by separate workers with the same water sample, owing to slight differences of media and variations in technique.

That such differing results are possible does not invalidate all quantitative work, but they serve to emphasize strongly the necessity for uniformity of method and procedure, while they indicate equally emphatically the need for stating the precise conditions under which any given series of results was obtained. It does not especially matter that only a proportion of the bacteria in a sample of water develop into colonies on the plates made, if that proportion is a constant one. Only relative results are required.

With media of uniform composition and reaction, used

according to a definite procedure, the results obtained are sufficiently comparable to yield results of importance, and under such circumstances simple enumeration methods have their place and their value, although they now only occupy a quite secondary place, and one accessory to more important methods of examination.

It should be mentioned that a considerable number of bacteriologists still regard fine adjustments of the reaction of media as of relatively small importance, holding that even when using exactly the same medium different bacteriologists would obtain with the same water very different results. Further, that as the results derived from the numerical counts of bacteria in a water can only be utilized in the broadest way, slight variations in alkalinity and the like are of quite secondary importance.

The figures obtained in a quantitative examination of water are influenced not only by the composition of the medium, but also by the physical conditions of collection. For example, the number of organisms in the middle of a stream will be somewhat different from the number near the sides.

The influence of sedimentation has been explained in Chapter I., so that it is obvious that the water entering a large reservoir will contain, under ordinary conditions, more organisms per c.c. than the water leaving it.

Of particular importance is the influence of previous rainfall. Heavy rains will markedly increase the number of organisms in moorland streams, owing to the washing in of soil organisms.

Delépine (Delépine, 1898), amongst others, has drawn attention to this, and has shown that moorland streams which in dry weather contained 40 to 80 organisms per c.c., developing on gelatine plates, during and after heavy rain contained from 200 to 280 per c.c. On the other hand, heavy rainfall, by diluting the water with a comparatively germ-free water, may greatly reduce the

number of organisms. In the case of surface wells the writer has found the influence of rainfall very variable, in some cases increasing the number of organisms, owing to the washing in of soil and other adventitious bacteria, in other cases lowering the germ content by dilution. The period of time elapsing between the rainfall and the examination is obviously one important influencing factor.

For well waters the duration of pumping has a considerable influence upon the bacterial content. Prolonged pumping causes a well to drain a larger area than gentle pumping, the area drained having the form of an inverted cone gradually rounding off to the nearly level subsoil water. In this way considerable variations in the analytical results may be obtained.

With these many and varied factors influencing the number of organisms which may develop upon agar and gelatine plates, it will be recognised that many of the published results, lacking, as they do, particulars as to the reaction of the medium used, time of incubation, method of enumeration, etc., are of but little value for comparative purposes, or as standards upon which to base deductions as to the number of organisms found in the different classes of waters. Plenty of such figures will be found in the older text-books and in special papers on the subject, but in view of these considerations the writer has refrained from making extensive use of them.

On the other hand, it is possible to have broad ideas on the subject.

Rain which has passed through dust-laden air will invariably contain some bacteria, the number varying with the amount of dust in the air and the cleanliness of the surface from which it is collected. The average number is usually small.

The number of organisms in upland surface waters will obviously depend upon the nature of the uplands, particularly whether cultivated or barren, and whether animals

graze upon them, while, as already mentioned, the influence of heavy rain is a variable and important factor. In pure moorland waters the number of organisms which develop at 37° C. is usually few—in the writer's experience from 0 to 30, and not usually more than 4 to 5—while the number growing in gelatine plates is more variable, varying from about 70 to 300 per c.c. These latter figures are based upon the following conditions: gelatine + 1 per cent. reaction, temperature of incubation 20° to 22° C., colonies counted by naked eye and after as long as possible (usually three days).

The number of bacteria in river water must obviously be subject to extreme variation, depending, as it does, on the amount of contamination, the rainfall, the temperature, and other factors. There are usually several thousand organisms developing per c.c. on gelatine plates. Some figures obtained for certain rivers are given in Chapter I., p. 5.

Even in rivers used for drinking purposes and at the different intakes, the organisms are frequently to be counted by thousands. Thus, for the river Severn at Shrewsbury an average number of 13,000 per c.c. is recorded at the waterworks by the Royal Sewage Commission.

Houston (Houston, 1906, *a*, and 1906, *b*), for the Thames at Sunbury (excluding the Staines supply, above the intakes for the whole of the Thames derived waters), for the river Lea at the intake for the East London Lea derived water, and at Hornsey above the intake of the New River water, obtained the following figures as an average for gelatine media (incubated at 20° to 22° C.; reaction not stated). All are raw unfiltered waters:

	Thames.	*Lea.*	*New River.*
November, 1905	1,633	3,946	718
December, ,, ...	740	2,050	621
January, 1906	2,075	5,192	1,455
February, ,, ...	1,679	3,083	1,304
March, ,, ...	1,161	1,308	291
April, ,, ...	277	471	149
May, ,, ...	1,064	1,350	352
June, ,, ...	382	598	198
July, ,, ...	952	1,190	450

Spring water and deep well water have a similar origin and bacterial content, and both should contain but few organisms—usually considerably less than 50 per c.c. on gelatine media incubated at 20° to 22° C.; while the number developing on agar plates at blood heat is frequently less than 1 per c.c. These waters are soil-filtered waters, and should contain very few bacteria, while the number is not subject to much variation.

Surface wells, on the other hand, usually contain a large number of bacteria, even when free from objectionable pollution, while the number present, even for the same well, and when the examinations are made within a short time of one another, is liable to very marked variation.

The number of bacteria varies markedly according to whether it is an open draw well, or one covered and provided with a pump, the numbers in the former case being frequently very large, and five, ten, or more times as numerous as in the latter cases; that is, comparing wells with a similar subsoil water.

The differences caused by rainfall and mechanical disturbances are very considerable, and often much greater than those caused by the access of bacteria from harmful pollution. It follows that simple numerical estimations are quite useless as guides to purity and safety for this class of waters.

The writer has recently completed the examination of over fifty different surface wells, many of them being examined a number of times. In the wells quite free from any contamination the agar (37° C.) enumerations were usually low and under 10 per c.c.; but in many of the less obviously pure supplies, which, however, from the other parts of the bacteriological examination could not be considered harmfully polluted, 100 or more colonies per c.c. were not infrequently found.

The gelatine counts for pure and contaminated wells alike showed extreme variation, the numbers per c.c. ranging from 100 or so to 20,000 or more.

Other Standard Media.

In view of the great difficulties inherent to the obtaining of ordinary nutrient gelatine and agar of uniform standard composition, some attention has been paid to the possibility of using media of simpler composition and preparation. In this connection simple solutions of gelatine and agar in water and Nährstoff agar may be mentioned.

Agar and gelatine, dissolved in distilled water instead of nutrient broth, have been employed to a limited extent; but sufficient data are not at present available to estimate their true value in water bacteriology. The English Committee on Standard Methods, in its report (1904), remarks: 'Since with a polluted water (detection of pollution being the ultimate aim in water examination) nutrient gelatine gives a relatively larger number of colonies than distilled-water gelatine, nutrient gelatine should be used when one gelatine only is employed. At the same time, it is recognised that cultures in distilled-water gelatine compared with cultures in nutrient gelatine often give useful indications. Thus, with an unpolluted water the number of colonies is usually relatively larger in distilled-water gelatine than in nutrient gelatine; with a polluted water the converse is the case. Therefore, the use of *both* gelatines (distilled-water and nutrient) is desirable, sets of plates being made with each medium.'

Gage and Adams (Gage and Adams, 1904) showed that the kind of water used in making up the medium had a considerable influence upon the number of bacteria developing upon that medium, so that these simple media must be made up with distilled water. They must also be brought to a uniform reaction.

Nährstoff agar was introduced by Hesse and Niedner (Hesse and Niedner, 1898). The special feature is the introduction of a new substance—an albumose obtained, under the commercial name of Nährstoff-Heyden, from

Heyden, near Dresden. The reaction of this material is neutral, and it dissolves completely in water. The nutrient medium has the following simple composition :

12·5 grammes agar.
7·5 grammes albumose (Nährstoff-Heyden).
1,000 c.c. distilled water.

The medium is said to require no correction for acidity or alkalinity. The inoculated plates are grown at room temperatures in the dark, and until fresh colonies cease to develop (two to three weeks). At least 10 c.c. of the medium is used for each plate.

The chief advantage claimed for it is that its simple composition and natural neutrality enables uniform results to be obtained and comparisons instituted with figures from other sources. The different workers who have reported results with this medium—Hesse and Niedner, Müller, Gage and Phelps, Gage and Adams, Prall, and Gustav Hesse—find that more organisms develop upon it than upon the ordinary nutrient media. As Müller (Müller, 1900) and Gage and Phelps (Gage and Phelps, 1902, *a*) point out, this is probably due to the growth of certain species of bacteria, which do not find the conditions suitable for their development upon the ordinary media. This is also confirmed by the later work of Gage and Adams (1904), in which they compared pure cultures of different bacteria on standard gelatine, agar, and Nährstoff agar, the highest counts being obtained (with the species studied) when gelatine media was used and the lowest with Nährstoff agar, showing that the increase is due to the presence of bacteria other than those commonly isolated. These workers also found that a reduction of the amount of the albumose (Nährstoff-Heyden) from 1·0 to 0·5 per cent. resulted in a considerable increase in the bacterial counts for the various waters studied.

This medium has the advantages of ready preparation

and uniform composition, and as such is of value for comparative purposes, but it possesses the disadvantage that owing to the great increase which takes place in the number of colonies of certain classes of natural water bacteria, it brings out the difference, as Müller and Prall (Prall, 1902) point out, between pure and polluted waters much less distinctly than ordinary nutrient gelatine and agar. This is shown by the following table, taken from Gage and Phelps' careful paper:

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON STANDARD AGAR AND ON NÄHRSTOFF AGAR FOR DIFFERENT CLASSES OF WATERS.

	Number of Days' Incubation.						
	2.	3.	4.	5.	6.	7.	8.
STANDARD AGAR:							
Ground water	0	5	6	6	6	6	6
Filtered water	6	7	7	7	7	7	7
Merrimac River	6	7	7	8	8	9	9
Filtered sewage	14	17	18	19	19	19	19
Sewage	34	44	46	46	46	46	46
NÄHRSTOFF AGAR:							
Ground water	6	43	78	88	93	100	100
Filtered water	37	69	80	92	98	100	100
Merrimac River	29	78	93	97	97	99	100
Filtered sewage	26	65	93	95	97	99	100
Sewage	39	75	95	100	100	100	100

This table shows that with Nährstoff agar, after eight days' incubation, the number of organisms in the purer waters was as great as with sewage, although well-marked differences are shown with ordinary agar. On this ground it cannot be considered as a suitable medium to replace ordinary gelatine and agar.

The Effect of Sand Filtration on the Bacterial Content.

It is well recognised that the purification of water by filtration through sand is only to a very minor degree mechanical; it is mainly vital and biological. Sterilized sand, as was shown first by Piefke, does not remove the bacteria from water; indeed, an increased number is present during the first few days of filtration. Within a day or two of starting the filtration of a natural water through sand, a slimy, more or less gelatinous layer is formed on the surface. This slimy layer is composed largely of bacteria, algæ, and other lowly forms of vegetable life. Its composition varies to a certain extent with the kind of water used.

It is this gelatinous layer which is the essential factor in the filtration processes, the action of the deeper layers being comparatively trivial. A sand filter freshly started acts merely as a mechanical strainer, and only when this layer is sufficiently developed does it act efficiently.

Such a filter-bed is capable, under suitable circumstances, of removing the vast majority of bacteria from water. The percentage number of organisms removed will depend upon a number of factors. The kind of water treated exerts a considerable influence upon the composition of the gelatinous layer and the rate of filtration. If it is rich in organic matter, the filter becomes more rapidly clogged, and filtration becomes very slow. The size of the sand grains is a factor, fine-grained filters yielding a more uniform result and a more rapidly formed filtering layer. The depth of the sand is not of great importance, but it must not be less than 30 centimetres (1 foot), according to Koch. The two chief factors, however, which influence the precentage purification are the rate of filtration and, particularly, the age of the filter.

If water is passed rapidly through a filter, the percentage

of bacteria removed will diminish. Koch recommended that the rate should not exceed 100 millimetres (4 inches) per hour. Slow is safer than rapid filtration.

The importance of the age of the filter is, in the main, a question of the thickness of the gelatinous layer. With time this slimy layer gradually increases in thickness, the rate at which this takes place depending upon the amount and character of the water filtered. With increased thickness the rate of filtration steadily diminishes until it becomes extremely slow. The sand has then to be cleaned, a certain amount of the surface layers being removed. This removal and the mechanical disturbance destroys to a considerable extent the bacterial efficiency, and when it is used again an excessive proportion of the micro-organisms in the water pass through. It is frequently necessary to waste the water for several days until normal filtration is re-established. Apart from certain exceptional cases, filter-beds must be worked intermittently.

The percentage of organisms removed should be 98 or more, and Koch's original standard that the effluent from each filter should not yield more than 100 micro-organisms per c.c. on gelatine plates, is generally accepted as one which should not be exceeded for satisfactory filtration.

For the six months ending April 30, 1906, Houston (Houston, 1906, *b*) found that the average number of microbes in the Metropolitan water-supply (after sedimentation and filtration) was 9·8 per c.c.

It is very necessary not only that the mixed filtered water be tested, but that each separate filter-bed should be bacteriologically examined.

The examinations, to be of use, must be made frequently and systematically; a daily determination should be aimed at. In all cases in which the number of bacteria present is above the average, a careful investigation of the faulty bed, or beds, should be made.

In addition to estimating the actual number of organisms

developing in the filtered water, the percentage of bacteria removed by filtration was determined by the Massachusetts State Board of Health.

A further advance was made by this Board when during the last few years it caused to be made careful investigations, at the Lawrence Experimental Station, upon the percentage of *B. coli* removed by sand filtration. Thus in the 1899 report, at the Lawrence City Filter, 180 samples of the river water were examined before and after filtration, and 99·5 per cent. of the *B. coli* were removed by the filtration. In the 1900 report they had come to the conclusion that the removal of *B. coli* is a better means by which to judge of efficient hygienic filtration than a determination of the total number of bacteria present.

In the examination of filtered water the number of *B. coli* present should be determined in every case, in addition to the number of organisms on gelatine and agar plates, and the percentage removed should also be ascertained by careful examinations of the unfiltered as well as the filtered water.

CHAPTER III

Bacteriology of Excreta in Relation to the Bacteriological Examination of Water

THE main object of the bacteriological examination of water is to ascertain whether sewage and excreta have gained access to it, directly or indirectly. It is, therefore, obviously of the utmost importance to have a knowledge of the organisms prevalent in excreta and of their relative abundance. While it is of value to have reliable data as to the presence in fæces of such specially pathogenic organisms as the bacilli of typhoid fever and dysentery, yet, as a means of detecting the presence of excreta in water-supplies, a knowledge of the organisms most numerous and widely prevalent in ordinary *healthy* stools is of far greater importance, since the detection of these in waters can serve as an indication of the presence of excreta.

Houston (Houston, 1902-1903, *a*) examined seventeen normal stools of healthy persons. The average results obtained per gramme of fæces were as follows:

Total number of bacteria (gelatine at 20° C., and agar at 37° C.) between 100 million and 1,000 million.

Spores of aërobic bacteria between 1,000 and 10,000.

B. coli between 100 million and 1,000 million.

B. enteritidis sporogenes spores (based on milk test) between 1 million and 10 million, and about the same number of virulent organisms.

Streptococci at least 100,000.

In a subsequent report Houston remarks that later work leads him to regard this number of streptococci as probably an under-estimate, and he states that in some samples of fæces the streptococci may exceed the *B. coli* in number.

Ninety-one per cent. of the streptococci formed short to medium chains in broth cultures, 81 per cent. produced clotting in milk, while practically all were either non-pathogenic to mice, or their pathogenicity was open to some degree of doubt.

The great majority of the *B. coli* were quite typical in their characters.

Subsequently Houston (Houston, 1903-1904) specially investigated the presence in fæces of non-lactose fermenting bacteria of the colon type (*i.e.*, bacilli like *B. coli* in many of their characters, but which do not ferment lactose). Out of 257 colonies isolated from 12 stools, no less than 251 produced acid and gas in a lactose-peptone medium, showing how scanty non-lactose-fermenting bacteria of this type are in healthy fæces, a point of much significance. Three hundred streptococci were also isolated, and found to comprise a large number of different varieties, with very varying vitality and viability outside the animal body.

Ford (Ford, 1903) made a careful study of the aërobic bacteria found in the different parts of the human alimentary canal in a number of autopsies, selected regardless of the morbid conditions present, and made within a few hours after death. Nearly 700 cultures were obtained from the 50 autopsies studied. The organisms were found to belong to practically 50 distinct species of bacteria. Of the 700 organisms, 200 were typical *B. coli*, including under that head both the saccharose (the *B. coli communior* of Durham) and the non-saccharose fermenters.

Nearly 200 other cultures were typical *Bacterium lactis aerogenes*, most of them fermenting glucose, saccharose, and lactose alike, and representing the main type of this species.

For a similar organism, except that it does not ferment saccharose, Ford suggested the name *Bacterium duodenale*.

Ford found that these four species (*i.e.*, the two last and the two varieties of *B. coli*) were the most frequently encountered and the most widely distributed of all the aërobic intestinal bacteria, and at one time or another occupied every region of the bowel. The next most frequently present forms were *B. vulgaris* (a variety of *Proteus vulgaris*) and *B. cloacæ*.

As regards their distribution, the two varieties of *Bacterium lactis aerogenes* were found in practically every stomach examination made, and may be considered the chief normal inhabitants of that organ, *B. coli* being only occasionally present. In the duodenum *Bacterium lactis aerogenes* was still the predominant form, but the number of *B. coli* was considerably increased, while members of the proteus group were numerous here. In the cæcum this relative abundance was reversed. In the rectum *B. coli* was especially abundant. This was also the favourite seat for *B. alcaligenes*, while *B. pyocyaneus* was more frequently found in this region than in any other. Both the proteus and the cloacæ groups diminished in numbers in the passage through the large intestine. Ford's work contains a careful description of the characters of all the bacilli isolated, and is of interest as showing the organisms commonly present in the alimentary canal. It does not follow, and it would not be true to conclude, that all these organisms are proportionately represented in fæces. The water bacteriologist is, of course, mainly concerned with the bacteria voided.

MacConkey (MacConkey, 1905) examined twenty-three samples of human fæces, from which 241 lactose-fermenting organisms were isolated. They all had the following characters: They were non-sporing, Gram negative, facultative anaërobic bacilli, giving gray-white growths on agar and gelatine without liquefaction of the latter, producing acid and clot in milk, general turbidity with formation of

indol in broth, and fermenting glucose, lactose, and mannite with the production of both acid and gas. He classified them into four groups as follows:

Group I.: Do not ferment either cane-sugar or dulcite; 83 organisms, or 34 per cent. of the whole.

Group II.: Do not ferment cane-sugar, but ferment dulcite; 93 organisms, or 38 per cent. of the whole.

Group III.: Ferment both cane-sugar and dulcite; 36 organisms, or 15 per cent. of the whole.

Group IV.: Ferment cane-sugar, but not dulcite; 29 organisms, or 12 per cent. of the whole.

Only four organisms of the 241 were shown definitely to belong to the *Bacterium lactis aerogenes* type, while *B. cloacæ* was not isolated in a single instance.

There are very few data available to say how far the intestinal flora is influenced by the class of food taken. Hammerl (Hammerl, 1897, *a*) concluded that the food, whether it was germ-free or not, vegetable or mixed, had no decisive influence upon the number of bacteria in the fæces. When the food was sterile the usual saprophytes disappeared from the stools, and plates showed organisms of the coli and lactis aerogenes groups in pure culture.

While pathogenic conditions may, to a certain extent, modify the bacterial content, it is probable that the organisms most abundantly present in disease are the same as those met with in healthy stools. Certainly this is true in the case of typhoid fever.

Bacteriology of Animal Excreta.—It is obviously of much importance to ascertain whether the excreta of the ordinary domestic animals have bacteriological characters similar to those of man. This subject has been investigated by a number of workers.

Dyar and Keith (Dyar and Keith, 1893) examined the excreta of horses, goats, rabbits, cats, dogs, pigs, and cows, and found *B. coli* in all, but only in small numbers in goats

and rabbits. They found complete agreement between the characters of these *B. coli* and those isolated from man. In the horse they described a new species, *B. equi intestinalis*, as a regular, and the most commonly present, inhabitant of the intestines.

The presence of *B. coli* in the excreta of many animals was soon confirmed by other workers, Fremlin, Brotzu, Theobald Smith, etc. Moore and Wright (Moore and Wright, 1902) examined the large and small intestines of freshly killed animals, comprising 9 horses, 11 cattle, 8 sheep, 7 pigs, 6 dogs, and 3 chickens, and isolated *B. coli* from them all, but could not find this organism in the intestine of a number of frogs. They found that the pathogenic power of the cultures from the different species of animals varied considerably. The colon bacilli from dogs were pathogenic when injected intraperitoneally into guinea-pigs; when derived from other animals they usually did not cause a fatal result.

Heinick (Heinick, 1903) investigated the intestinal contents of twenty-three pigs. He found *B. coli* as the most numerous organism, and stated that they differed in no way from those derived from human sources. *Bacterium lactis aerogenes* was found by him to be almost equally numerous. Inoculation of traces of the intestinal contents into mice usually yielded negative results, only two out of sixty-five dying within the two and a half days taken as a time limit. From his investigations he concluded that bacteria pathogenic to animals were especially few in the intestinal contents of pigs.

Houston (Houston, 1903-1904) examined eight gulls and two guillemots. He found that all the gulls contained typical *B. coli* in their excrement in enormous numbers, and that the guillemots did not contain *B. coli* or its varieties. Streptococci and spores of *B. enteritidis sporogenes* were present in two of the gulls in small numbers, but absent in the others, and in the two guillemots, in the amounts

examined. Houston ascribes the differences met with to the different habits as regards food of these sea-birds. Gulls are prone to feed on garbage, while guillemots are said to be clean feeders. The *B. coli* in the gull were indistinguishable from those obtained from the human intestine. The same observer examined for *B. coli* a number of fish and crustaceæ 'dredged from the sea in localities to which, on topographical considerations, no reasonable objection could be taken on the score of pollution.' In five experiments typical *B. coli* were isolated, in twenty atypical coli-like microbes were obtained, and in the remaining thirteen the results were quite negative. In the five positive results in which typical *B. coli* were obtained (from whiting, crab, hermit crab, dab, and sole) either the whole fish or the whole of the alimentary tract had been added to the nutrient medium. Houston concluded that typical *B. coli* are either absent, or present in small numbers only, in the intestines of fish dredged from the sea in localities remote from sewage pollution, and that, inasmuch as 34 per cent. of the experiments were wholly negative, it can hardly be the case that even coli-like microbes are present naturally in abundance in fish.

Eyre (Eyre, 1904, *a*) states that he readily isolated *B. coli* from the intestinal canal of the mouse, rat, guinea-pig, rabbit, cat, dog, sheep, horse, goat, cow, the common fowl, duck, pigeon, and sparrow. He also examined two sea-birds, a black-headed diver and a common gull shot three miles from land, and found *B. coli* to be the predominant organism present in the intestinal contents. In a batch of fish freshly caught off the Lincolnshire coast, *B. coli* was isolated in varying numbers from the intestinal contents of all the fish.

Johnson (Johnson, 1904) examined sixty-seven freshly caught fish from the Illinois and Mississippi Rivers. Immediately upon their arrival at the laboratory they were opened, and tubes of broth containing Parietti's solution

were inoculated from the contents of the large intestine and stomach of each fish. *B. coli* were isolated from forty-seven fishes. In twenty-four cases they were found in the stomach, and in forty-one in the intestine. No information as to the bacterial condition of the river water was recorded.

MacConkey (MacConkey, 1905) examined 25 samples of animal excreta, isolating 239 lactose-fermenting organisms. The excreta examined comprised 5 specimens from the horse, 6 from cows, 3 from rabbits, 1 from a monkey, and 14 from a cat on special diet. He classified the isolated lactose-fermenting organisms into the four groups recorded above (p. 30). In the following table his results are set out classified into these four groups, including the organisms from both human and animal sources :

Group.	Fermentation of		Number of Organisms isolated.			Results as Percentages.		
	Saccharose.	Dulcite.	Human.	Animal.	Total.	Human.	Animal.	Average.
I.	−	−	83	37	120	34	15	25
II.	−	+	93	85	178	38	36	37
III.	+	+	36	74	110	15	31	23
IV.	+	−	29	43	72	12	18	15
			241	239	480	100	100	100

From his work MacConkey concluded that 'at present there is no means of differentiating the lactose-fermenting organisms of human from those of animal origin; or those of normal dejecta from those found in enteritis.' This investigator also showed that the proportion of these groups altered with alteration of foodstuffs, and that when a cat was fed only upon cooked food, Group I. disappeared from the excreta.

During part of 1904 and 1905 the writer made an extended investigation of human and animal excreta, the object in view being to study the possibility of obtaining bacterial

evidence which would offer means by which excreta from human and from animal sources could be readily distinguished. Numerous *B. coli* and allied organisms were isolated and investigated as regards their action upon lactose, dulcite, mannite, raffinose, glycerine, maltose, galactose, lævulose, saccharose, starch, cellulose, etc. It was hoped that the use of starch and cellulose would be of especial value in differentiating the excreta of herbivora. There were examined the dung of 3 pigs, 2 cows, 5 horses, 3 sheep, the contents of the large intestine (immediately after slaughter) of 11 pigs, 2 cows, 1 calf, and 3 bullocks, as well as a number of samples of healthy human fæces, 3 stools from cases of infantile diarrhœa, and 2 from cases of typhoid fever. Although considerable differences were met with, it was not found possible to ascribe certain groups of organisms to certain animals, and at present there is no reliable means of distinguishing between the *B. coli* organisms derived from animal excreta and those from human excreta.

The above investigations, while amply demonstrating the presence of the *B. coli* group of organisms in animal excreta, give no information in regard to streptococci and *B. enteritidis sporogenes*.

A number of quite fresh specimens of excreta were recently examined by the writer to investigate the prevalence of these organisms. The results are shown in the table on page 35.

This table shows that *B. coli* is abundant in horse, cow, sheep, and pig excreta; that streptococci are very numerous in horse, cow, and sheep dung, but absent in pig excreta ($\frac{1}{10}$ gramme); and that spores of *B. enteritidis sporogenes* are regularly met with in all, although much less abundant.

The relative proportions of streptococci and *B. coli* varied somewhat; in general they were nearly equally numerous in horse, cow, and sheep excreta. Spores of *B. enteritidis sporogenes* were especially abundant in pig excreta. These

results, taken as a whole, show that streptococci and *B. enteritidis sporogenes* are also widely distributed in animal excreta.

In 1904 numerical estimations by the writer of three samples of pig excreta showed *B. coli* between 100,000 and 1,000,000 per gramme in two samples, and over 1,000,000 in the third, but no streptococci were found in 0·001 gramme or less.

B. coli seems to be present in the dejecta of most, if not of all, mammals, while it is also numerous in the excreta of other animals. Up to the present, although differences between the relative proportions in which the different sugar fermenters are present can be demonstrated, it is not possible to say, from its biological characters, that a given *B. coli* isolated from water is derived from one animal more than another.

Source.	*B. Coli.*	Streptococci.	*B. enteritidis sporogenes* Spores.
	Approximate number per Gramme of Excreta.		
Horse No. 1	over 1 million	$\frac{1}{10}$ to 1 million	10 to 100
,, No. 2	$\frac{1}{10}$ to 1 million	over 1 million	100 to 1,000
,, No. 3	1,000 to 10,000	over 1 million	100 to 1,000
Cow No. 1 ...	$\frac{1}{10}$ to 1 million	10,000 to 100,000	100 to 1,000
,, No. 2 ...	10,000 to 100,000	$\frac{1}{10}$ to 1 million	10 to 100
,, No. 3 ...	1 to 10 millions	over 10 millions	10 to 100
,, No. 4 ...	1 to 10 millions	$\frac{1}{10}$ to 1 million	100 to 1,000
Pig No. 1 ...	over 100 millions	absent	$\frac{1}{10}$ to 1 million
,, No. 2 ...	10 to 100 millions	absent	10,000 to 100,000
,, No. 3 ...	70 millions	absent	1,000 to 10,000
Sheep No. 1	10 to 100 millions	1 to 10 millions	10 to 100
,, No. 2	10 to 100 millions	10 to 100 millions	10 to 100

CHAPTER IV

The Bacteriology of Sewage in Relation to the Bacteriological Examination of Water

CRUDE sewage is a fluid of great bacterial complexity, and contains a vast number of organisms. Naturally, for such a complex fluid, the number of different kinds of bacteria which may be isolated from it is very large, and some of the species are probably peculiar to the particular local sewage investigated. Careful enumeration has, however, clearly shown that certain organisms—or, more properly speaking, certain well-defined groups of organisms—are not only invariably present, but are present in very large numbers.

From the point of view from which this chapter is written, it is these organisms which are of paramount importance, and the large number of other bacteria which have been isolated from time to time from individual samples, and whose numerical distribution is unknown, are of little practical significance.

The most prolonged and extended work on this aspect of the bacteriology of sewage stands to the credit of Houston, his various findings being contained in the Reports of the Royal Commission on Sewage Disposal, Reports to the London County Council, and in the Annual Reports of the Medical Officer of the Local Government Board.

In one of his more recent reports Houston (Houston,

1904, *b*, p. 210) gives the following average figures for domestic and ordinary mixed sewage:

Total number of bacteria (gelatine at 20° C.): More than 10,000,000, but less than 100,000,000, bacteria per c.c.

Total number of bacteria (agar at 37° C.): More than 1,000,000, but less than 10,000,000, bacteria per c.c.

B. coli (and closely allied forms): About 100,000 in 1 c.c.

Indol producing bacteria: About 100,000 in 1 c.c.

Spores of *B. enteritidis sporogenes:* 100 to 1,000 per c.c.

Bile-salt broth test and neutral-red broth test: Usually a positive result with $\frac{1}{100000}$ c.c.

In an earlier report (Houston, 1902, *a*) the number of streptococci in crude sewage is given as at least 1,000 per c.c. It is also stated that 0·001 c.c. of crude sewage usually produces gas in gelatine shake cultures in twenty-four hours at 20° C. Also that the subcutaneous injection of crude sewage, even in moderate quantity, into rodents, is always followed by a local reaction, and not uncommonly leads to a fatal result.

As regards the number of typical *B. coli* among these 100,000 *B. coli* and closely allied forms per c.c., Houston elsewhere records that 65 to 85 per cent. of them possess characters which he describes as flaginac (see p. 225 for explanation of the term), and this figure may be taken as about the percentage proportion of typical *B. coli.*

Other workers have also investigated the bacteriology of crude sewage. Thus, H. Chick (Chick, 1900) found the sewage at West Derby to contain 27,000 to 50,000 *B. coli*, Manchester sewage 5,000 (mean of five determinations), and that of Leeds 19,700 (mean of six determinations). She, however, used a phenol-lactose-agar medium, which probably had a restraining influence upon the development of this bacillus.

In America, Clark and Gage (Clark and Gage, 1902)

found these organisms very abundant in Lawrence crude sewage. They state that 'examinations of sewage at the experimental station show that in dilutions of $\frac{1}{10000}$ we may expect to find *B. coli* in 95 per cent. of the samples, and that we may identify the sewage streptococcus in 67 per cent. of the samples.

'A dilution of $\frac{1}{50000}$ cuts the percentage of positive tests for *B. coli* to 78 per cent. of the samples, and the percentage of streptococcus to 6 per cent. of the samples. In dilutions of $\frac{1}{100000}$, *B. coli* was found in 40 per cent. of the samples, and in a dilution of $\frac{1}{1000000}$, *B. coli* was found in 6 per cent. of the samples. The sewage streptococcus could not be identified in dilutions greater than $\frac{1}{50000}$. *B. enteritidis sporogenes* can usually be detected in dilutions of $\frac{1}{500}$ to $\frac{1}{1000}$, but in dilutions greater than this it is only very rarely detected.'

These figures show that in both English and American sewage *B. coli*, streptococci, and *B. enteritidis sporogenes* are abundant—results which have been repeatedly confirmed, and which are in accordance with the general experience of bacteriologists. In addition, a number of special organisms, apart from those just mentioned, have been isolated from sewage, and their characters described by different workers, particularly by Jordan, Laws and Andrewes (1894), and Houston.

Jordan (Jordan, 1890) described a number of named varieties, one of which—*B. cloacæ*—is stated to be one of the commonest organisms in Lawrence sewage. The characters of this bacillus are considered on p. 88.

Houston (Houston, 1899) isolated and described some of the bacteria found in London crude sewage and in the effluents from the coke-beds. Of these bacteria, the **sewage proteus** was stated to be one of the most abundant organisms in London crude sewage, frequently present as many as 100,000 per c.c. Its characters were as follows:

A small, actively motile bacillus; no spores.

Grows readily on gelatine media, and rapidly liquefies it.

The gelatine colonies are circular, and show rapid liquefaction. No 'swarming colonies.'

Produces abundant gas in gelatine shake.

Slimy, yellowish-white growth on potato.

Uniform turbidity in broth.

Grayish-white growth on agar.

Produces acid in litmus-milk, with or without coagulation.

Reduces nitrates to nitrites vigorously.

Produces no indol.

A member of the large proteus group, but differs from *Proteus vulgaris*.

The number of different species of organisms in sewage is very great, and it is highly probable that many of them occur in all specimens of ordinary sewage; but, except for *B. coli*, streptococci, and *B. enteritidis sporogenes*, their presence has not been ascertained with sufficient constancy, nor has their numerical occurrence been sufficiently investigated, to make them of value as indicators of sewage pollution. Further prolonged investigation is required.

The bacterial composition of sewage will naturally to a certain extent vary with its source and the general proportions of the different constituents which make up the sewage. Obviously, the dilution of the sewage will have a direct influence upon the numerical presence of these bacterial indicators in sewage, while in the case of sewages containing large quantities of trade waste the presence of these added chemicals will affect the bacterial content.

Still, speaking broadly, the comprehensive work of Houston has established the fact that the average numerical bacterial composition given by him is true for all kinds of mixed sewages.

Effects of Sewage Treatment upon the Presence of Bacterial Indicators.

From the point of view of the bacteriological examination of water, it is of practical importance to inquire as to the effects of sewage treatment upon those especially abundant organisms which are used as indicators of sewage contamination. The reports of the Royal Commission on Sewage Disposal have made it abundantly clear that while the different processes for treating sewage may effect their immediate purpose of producing a non-putrefying effluent, yet they never yield one which is sterile. The bacteriologist should be in a position to recognise when such effluents are gaining access to water-supplies, and for this purpose a knowledge of the bacterial content of sewage effluents is necessary.

Effluents from Sewage Farms.—Houston (Houston, 1904, *b*) made a careful investigation for the Royal Commission on Sewage Disposal of the biological qualities of the effluents from sewage farms. An abstract of some of his more important results is shown in the table on p. 41.

Compared with the original sewages, all the effluents exhibited a high percentage degree of purification; but, apart from a reduction in number, they showed no very appreciable biological modification. Houston remarks that sometimes the relative number of spores of *B. enteritidis sporogenes* was reduced, and that there was some evidence, especially in the better class of effluents, of a greater proportionate reduction, as compared with crude sewage, in the number of microbes growing at blood heat, over those growing at 20° C. Also on several occasions failure was experienced in isolating streptococci from the effluent.

In general, however, as Houston very definitely points out (p. 169), 'the results conclusively show that the treatment of sewage on land cannot be relied on *materially to modify* the potentially dangerous qualities of crude sewage.

Sewage Farm Effluent.	Soil of Farm.	Kind of Sewage.	Gallons of Sewage treated per Acre per 24 Hours.	Number of Organisms per c.c.		Average Number of *B. coli* per c.c.	Average Number of *B. enteritidis sporogenes* per c.c.
				20° C.	37° C.		
Aldershot camp...	sandy	domestic	25,000	183,300	37,300	1,000 to 10,000	10 to 100
Altrincham ...	peaty and sandy	domestic	46,000	363,400	7,300	100 to 1,000	1 to 10
Cambridge ...	sandy loam	mainly domestic	121,600	711,500	78,300	1,000 to 10,000	10 to 100
Beddington (Croydon) ...	gravelly loam	domestic	57,100	1,413,000	112,000	1,000 to 10,000	10 to 100
Leicester... ...	stiff clayey soil	mixed	21,500	532,800	70,500	1,000 to 10,000	10 to 100
South Norwood...	clay	domestic	12,000	778,300	31,100	100 to 1,000	10 to 100
Nottingham ...	light sandy loam	mixed	23,300	very variable; frequently less than 1,000	very variable; frequently less than 1,000	less than 10	less than 1
Rugby	heavy loam	mainly domestic	42,800	637,000	81,500	1,000 to 10,000	10 to 100

The actual number of objectionable microbes persisting in the effluents is too great to allow of much stress being laid on the great percentage reduction effected in the total number of microbes by the land treatment, or to insure any certainty that effluents from land processes are "relatively safe."'

Effluents from Biological Processes.— Houston extensively investigated this class of effluents. He sums up the general outcome of the experiments as follows (Houston, 1902, *a*, p. 26): 'The effluents from septic tanks, intermittent contact beds, continuous filtration beds, etc., contain an enormous number of bacteria. In some cases the percentage reduction of microbes in effluent as compared with raw sewage is striking. But as an effluent must be judged by the actual state it is in, and as the number of micro-organisms still remaining is nearly always very large, percentage purification would seem to be of minor importance. In not a few cases the bacteria are practically as numerous in the effluent as in the raw sewage. The different kinds of bacteria and their relative abundance appear to be very much the same in the effluents as in the crude sewage. Thus, as regards undesirable bacteria, the effluents frequently contain nearly as many *B. coli*, proteus-like germs, spores of *B. enteritidis sporogenes*, and streptococci, as crude sewage. In no case, seemingly, has the reduction of these objectionable bacteria been so marked as to be very material from the point of view of the epidemiologist. No definite proof has been furnished that the effluents from bacteria beds are conspicuously more safe in this sense in their possible relation to disease than is crude sewage. Indeed, all the available evidence tends to show that they must be regarded as nearly, if not quite, as dangerous to health as raw sewage. . . . The inoculation of animals with the effluents from bacterial beds seems to show that they are nearly as pathogenic as crude sewage.'

The extended series of experiments made for the

Massachusetts State Board of Health, to study the biological results of different methods of sewage treatment, including sand filtration processes, are also of much interest. As illustrating some of the results obtained, a few of the figures recorded in the report for the year 1904 (Massachusetts State Board of Health, 1904) may be given. It was found that while there was a marked reduction in the total number of bacteria, and also of the bacteria of non-sporing type by passage through the septic tank and through contact and continuous-intermittent or trickling filters, there was no perceptible reduction in the number of spore-bearing bacteria. The percentage removal of *B. coli* during treatment by different methods showed extensive variation. The septic tank investigated removed an average of 46 per cent. of the *B. coli*, the results varying from 0 to 71 per cent.

The sand filters removed 97 to 100 per cent. of the *B. coli*, and this whether treated with raw or septic sewage. Of the contact filters operating with raw sewage, one removed 49 and the other 71 per cent. of the *B. coli*, while a third, treated with septic sewage, removed 76 per cent. Both the trickling filters, composed of coarse materials, and treated with raw sewage at high rates, showed high percentage removal of this organism—*i.e.*, 97 and 99 per cent. respectively.

With regard to *effluents from chemical processes*, we are at present without sufficient data as to their precise biological composition, and as to how far any chemical process is capable of yielding a bacteriologically pure effluent.

These results are incorporated to show that from a bacteriological aspect sewage effluents are usually highly impure, and contain the same bacterial indicators as the original sewage, and that by the presence of these organisms the addition of sewage effluents to water-supplies may be detected with nearly the same facility as that of crude sewage itself. In the cases where the effluents are so purified that

these methods of determination are not available, present knowledge is sufficient to enable us to affirm that for practical purposes the addition of such effluents to water-supplies is harmless.

The experiments of Houston upon the inoculation of sewage with *B. pyocyaneus* are of great interest. This observer (Houston, 1904, *a*) added *B. pyocyaneus* in very large numbers to sewage before treatment—(1) in a continuous filter; (2) in a septic tank, followed by treatment in contact-beds. In the case of the continuous filter-bed, this organism appeared within less than ten minutes from the start of the experiment, and was present in the effluent for a considerable time afterwards — in Experiment 1 for at least five hours, and in Experiment 2 for ten days. In the case of the septic tank and contact-bed, *B. pyocyaneus* appeared in the septic tank liquor within two and a half hours from the start of the experiment, and in the contact-bed effluents at the earliest possible time—*i.e.*, at the first emptying of the bed after the passage of *B. pyocyaneus* through the septic tank and into the beds. In the first experiment this organism was still present in the septic tank liquor twenty-four hours from the start of the experiment, and in the contact-bed effluent up to the third day. In the second experiment this organism was isolated from the septic tank effluent and also from the contact-bed effluents on the ninth day. In all cases, however, it ultimately died out, and apparently could not exist for any length of time in the filters or in the tank.

Houston points out that these experiments seem to indicate that a proportion of the total number of microbes contained in each unit of sewage may escape prolonged exposure to the influences at work in these purification processes, and these bacteria can hardly be expected to undergo any marked modification in their biological characters during their brief sojourn in the beds or in the septic tank. In other words, the treatment would not

necessarily suffice for the certain destruction of pathogenic bacteria, and we must therefore regard the effluents from such processes as potentially dangerous. The use of indicators for the detection of the addition of sewage effluents to water is therefore both justifiable and necessary.

The Fate of Pathogenic Organisms in Sewage.

This is of considerable importance in connection with water bacteriology.

Laws and Andrewes (Laws and Andrewes, 1894) investigated the vitality of typhoid bacilli in sewage. They used London crude sewage (from Barking or Crossness), sterilized either by heat alone or twice filtered through a Berkefeld filter, and then heated to 60° C. in a sterile flask for twenty minutes on two consecutive days. They found that *B. coli* grew readily and multiplied abundantly in sterile sewage, but that *B. typhosus* quickly perished, and from their work concluded that sewage, even in the absence of the normal micro-organisms which it contains, is an unfavourable medium for the growth of typhoid bacilli, and that their death was probably only a matter of a few days or, at most, of a week or so.

They also tested the action of certain sewage micro-organisms upon the typhoid bacillus in filtered sterile sewage. The organisms selected were *B. fluorescens stercoralis*, *B. fluorescens liquefaciens*, *B. albus putidus*, and *B. cloacæ fluorescens*. Only the first of these bacteria seemed to have any marked effect upon the vitality of the typhoid bacillus, increasing the rate of its extinction. The admixture of all four organisms with *B. typhosus* appeared to have no effect in accelerating the death of the latter organism.

Klein (Klein, 1894-1895, *a*) investigated the vitality of *B. typhosus* and *Sp. choleræ* in sewage. He used sewage filtered through a Berkefeld filter and heated to 70° C. for fifteen minutes. This filtrate was used either alone or with the addition of 1 per cent. potassium nitrate. Flasks con-

taining the sewage were inoculated with agar cultures of the typhoid and cholera organisms respectively, and were kept at ordinary room temperatures. The experiments showed that the typhoid bacillus exhibited much greater vitality in the sterilized nitrate sewage than in simple sterilized sewage. In the latter, after some preliminary multiplication, they rapidly decreased in numbers, being alive, however, after eight weeks, while in nitrate sewage there was a marked increase in numbers, still considerable after eight weeks. The cholera vibrio similarly maintained itself much better in nitrate sewage, in one experiment being found alive after eight weeks, while in ordinary sterile sewage the vibrios died out between the eighth and twenty-fifth day.

Horrocks (Horrocks, 1899) studied the behaviour of the typhoid bacillus in sewage under varying conditions, a culture recently isolated from the spleen of a fatal case being used. The experiments were conducted at a temperature which varied between 16° and 22° C. The typhoid bacillus was found alive after sixty days' immersion in sterile sewage. The colonies of the bacillus on gelatine were somewhat altered and the resistance to carbolic acid was diminished, but the power of agglutination was unchanged. In the same way *B. coli*, after forty-two days in sewage filtered through a Berkefeld bougie, showed alteration of colony characters and diminished resistance to carbolic acid. When typhoid and colon bacilli were simultaneously inoculated into sterile sewage, the typhoid bacillus was isolated for five days, but not later.

Horrocks concluded that prolonged immersion in sterile sewage does not destroy the power of agglutination of the typhoid bacillus with typhoid serum. If the bacillus be still found, it will retain its specific characters.

MacConkey (MacConkey, 1902) investigated the longevity of the typhoid bacillus in both sewage and sewage effluents. Crude sewage from Leeds, and effluents from an open

sewage tank, from a Dibdin bed, and from a Cameron bed, were used. The experiments were conducted at room temperatures. For two, out of the four series made, unsterilized liquid was used, for the other two, incomplete sterilization of the sewage or effluent was carried out, about 600 c.c. being heated in a flask to 65° C. for from half to three-quarters of an hour. When cool the flasks were inoculated with *B. typhosus*.

In the partially sterilized sewage the typhoid bacilli gradually died out, their vitality, as was to be anticipated, being greatly influenced by the number of other organisms which remained alive. They were recovered thirteen days after inoculation, but not later, in one series, and after six days, but not later, in the other.

In the case of the crude unheated sewage inoculated with *B. typhosus*, this organism was recovered after thirteen days, but not later, in one series, while in the second series it could not be recovered, the first examination being made at the end of six days, and this although some 8,000,000 *B. typhosus* per c.c. were sown in the medium. MacConkey concluded that this organism does not multiply, but dies out more or less rapidly, in crude sewage.

In the partially sterilized effluents, the typhoid bacilli were recovered after ten and fifteen days in the sewage tank effluent, and after fifteen and ten days respectively in the Dibdin and Cameron effluents.

In the untreated effluents this organism was isolated after fifteen and seventeen days in the sewage tank effluent, and after a somewhat shorter time in the other.

MacConkey concluded that all these fluids are inimical to the growth of the typhoid bacillus. Also that typhoid bacilli can only find their way into sewage in small numbers, and that if they do reach the septic tanks or biological beds they are present in such small numbers, and the conditions are so adverse to their existence, that they will not survive the treatment. ‘ But if from any cause they arrived at the

beds or tanks in such numbers as the *B. coli*, then certainly they might appear in the effluent just as the *B. coli* does. But as in the case of the latter bacillus, so also in the case of the *B. typhosus*, there is no tendency to multiplication in the effluent.'

Russell and Fuller (Russell and Fuller, 1906) recently found that when the typhoid bacillus was exposed directly to the action of sewage bacteria its longevity was greatly diminished, three to five days being the longest time for which the organism could be recovered (for further particulars, see p. 102).

In regard to other organisms, anthrax bacilli have been found by Houston (Houston, 1902, *b*) in Yeovil sewage, both in the septic tank and in the primary and secondary coke-beds, also in the mud of the banks of the river Yeo. It is possible, and undoubtedly does occur, that anthrax bacilli gain access to waters which receive the sewage from hide and other factories dealing with articles which may be anthrax-infected. Such occasions are, however, very infrequent and local.

In unsterilized crude sewage, kept at room temperature, Houston (Houston, 1900) found that the cholera vibrios lived in one experiment for about four weeks, while in a second experiment they were not found after nine days.

CHAPTER V

Soil in Relation to the Bacteriological Examination of Water

THE bacteriology of water is intimately concerned with the bacteria in soil, an elementary fact which does not always receive the consideration it deserves. Except rain water, all natural waters come in contact with soil, and the bacteria in the soil, and the character of the soil, must, to a considerable extent, affect the flora of the water.

Surface soil usually contains a vast number of organisms. With increased depth the number rapidly diminishes, and below 4 to 5 feet but few bacteria are to be found in undisturbed soil. Houston (Houston, 1893), working with soil in the grounds of Morningside Asylum, Edinburgh, on a plot of land which was formerly a vegetable-garden, but which had lain untouched for some time, found, as the result of a large number of experiments, that the average number of germs in 1 gramme of soil was, on the surface, about 1,688,000; 1 foot deep, 1,100,000; 2 feet, 900,000; 3 feet, 174,000; 4 feet, 25,000; 5 feet, 920; and 6 feet deep, 410. These figures deal only with the numbers which will develop on gelatine media, and do not give any true idea of the total number of bacteria actually present, excluding, as they do, for example, the vast number of nitrifying organisms. They, however, illustrate the rapid decrease in the number of organisms which grow on gelatine media under aërobic conditions with increased soil depth, thus confirming the extended work of Fraenkel. In

Fraenkel's researches anaërobic bacteria were also found to be absent, or relatively absent, in the deeper layers.

The number also varies with the kind of soil, particularly whether virgin or cultivated. Thus, Houston (Houston, 1897-1898), who examined twenty-one samples of surface soil from different sources, found that the virgin sandy soils gave less than 100,000 bacteria per gramme, the other virgin soils about 1,000,000, the garden soils from 1,000,000 to 2,000,000, and the grossly polluted soils, in one case 26,000,000, and in the other 115,000,000, bacteria per gramme.

The number of spores relative to the total number of bacteria in soil is large, the proportion being often as high as from 1 to 10 to 1 to 3.

Bacteria from very varied sources are constantly being added to soil. Our knowledge of the bacteriology of soils is at present very limited, but we possess sufficient information to show that they have a considerable selective action upon bacteria. Certain organisms are found in almost all soils, others will only grow in certain soils and under certain conditions, while others tend to more or less rapidly die out in any soil. These differences depend not only upon the kind of organism, but also upon the physical conditions of the soil, such as temperature and moisture, while its chemical nature probably has considerable influence.

This selective action is of great importance in relation to the bacteriology of water, and, speaking broadly, we can recognise that soil has, to some extent, a bacterial flora of its own. Thus Fraenkel found *B. subtilis*, *B. mycoides*, and the 'hirn bacillus' as the three commonest species in the deeper layers, and Houston (*ibid.*, 1897-1898) found *B. myccides*, cladothrix, *B. fluorescens liquefaciens*, *B. fluorescens non-liquefaciens*, *B. subtilis*, and *B. mesentericus* commonly present in soil in considerable numbers. Of these he regards *B. mycoides* and cladothrix (Bismarck-brown cladothrix) as especially characteristic of, and abundant in, soil.

Thus, in one sample there were 99,000 *B. mycoides* per gramme of surface soil, and in nearly all the specimens examined it was present in great numbers (on an average about 32,000).

The Bismarck-brown cladothrix, identical with or closely allied to *Cladothrix dichotoma*, was also very abundant, on the average about 34,000 per gramme.

In a later investigation upon soil washings Houston (Houston, 1898-1899, *a*, and 1899-1900, *b*) confirmed these statements. He concluded that *B. mycoides* is commonly present in great numbers in soils, but is especially abundant in garden and pasture soils, and may be absent from or present in small numbers only, in pure peaty and sandy soils. He pointed out that pure potable waters do not contain *B. mycoides*, or contain it only in small numbers, and concluded that the presence of this organism in a potable water implies the presence of surface soil washings, not improbably from cultivated soil. To the presence of cladothrix he ascribed a similar significance as indicating soil contamination. He remarked that these two organisms may be met with in sewage, but only in very small numbers relatively to the total number of bacteria.

The essential characters of the Bismarck-brown cladothrix, as described by Houston, are as follows:

It consists morphologically of mycelial threads with apparent, but not real, branching. It grows very slowly in gelatine, and produces colonies of a brownish-white colour and of a tough, skin-like consistence. This skin becomes wrinkled and pitted as the growth, which is accompanied by slow liquefaction of the medium, proceeds. The gelatine round the colonies is stained brown. On potato the cladothrix grows slowly, producing minute colonies of a dirty brownish-white colour and of a tough consistence, which stain the surrounding medium a characteristic inky colour. In broth the growth is very slow, a mould-like appearance being produced; the nutrient medium gradually assumes a deep brown colour.

The organism can be readily identified by its characteristic colonies and brown staining on gelatine plates.

The chief characters of *B. mycoides* are as follows:

A large bacillus with rounded ends, chain formation frequent. Oval spores are formed rapidly on all media. Motile. Decolourized by Gram's method.

Gelatine Stab.—Growth takes place along line of inoculation with tree-like horizontal processes (arborescent growth). Fairly rapid liquefaction of the gelatine. Membrane forms on surface of the liquefied gelatine.

Agar Slope.—White, wrinkled, dry growth with fringe-like margins.

Broth.—Uniform turbidity; thick surface pellicle.

Potato.—Abundant white growth.

Gelatine Colonies.—White and circular when quite young, but soon irregular processes extend out rapidly into the gelatine. Gelatine liquefied.

Agar Colonies.—Superficial colonies at first circular, but rapidly send out characteristic root-like branchings.

No gas is produced in *glucose* media, and *lactose* is not fermented.

No indol produced.

B. mycoides also can be readily recognised by its characteristic colonies on gelatine and agar.

The Soil as a Filtering and Purifying Agent.

The filtering action of the soil is an important factor in the purification of water.

It forms, speaking broadly, an almost perfect filtering agent, the organisms being retained in its interstices. A consideration of what actually occurs around many surface wells will serve as a good illustration. Around the majority of these wells in country districts the ground is extensively manured or otherwise polluted. Rain washes the organisms derived from such contaminated material into the upper layers of the soil, where they are retained, and if the well

is properly covered and rendered impervious to a sufficient depth (so that a sufficient depth of filtering soil is obtained), they do not gain access to the water supplying the well.

If that were all, and the organisms so obtaining access to the soil could multiply there, or even if they could only keep alive, with constant additions from fresh deposition of manure or other polluted material, it would be only a question of time for these organisms to be washed through into the water-supply. In the soil, however, they have to compete with the natural soil organisms, and all the available evidence shows that although some of them can persist for long periods, there is little or no evidence of any multiplication, and ultimately they die out.

Some experiments of the writer (Savage, 1903, *b*) upon the self-purification of tipped refuse or 'made soil' illustrate the gradual replacement of the *B. coli* and other organisms, originally present abundantly in the tipped refuse, by *B. mycoides* and other soil organisms. In these experiments 'made soil' (the samples being collected at a depth of 2 feet) was examined, taken from deposits of known ages. In the samples deposited within two years *B. coli* was abundant, while the numbers of *B. mycoides* and cladothrix were either very few, or these organisms were absent altogether. In material deposited several years previously, on the other hand, no *B. coli* were found, while these soil organisms were very numerous. The soil organisms had replaced those in the original material.

This self-purification of soil is illustrated also on p. 60, where Houston's experiments on soil treated with sewage are considered.

It is obvious that if the rate of addition of these excretal organisms is not higher than the rate of purification, the soil can act for an indefinite period as an efficient filter, preventing the access of harmful organisms to the water-supply. The factors which can upset such an equilibrium are readily recognisable:

1. The addition of the excretal matter may be so abundant that the soil cannot purify itself rapidly enough, and becomes loaded with organisms, which are then washed into the water-supply.

2. The rainfall may be excessive, thus washing the bacteria through the soil into the subsoil water before the antagonism of the soil bacteria has had time to play its part.

3. The level of the ground water itself may be so high that its pollution occurs very readily.

4. The soil may be fissured, or otherwise incapable of retaining these adventitious organisms.

The character of the soil is certainly of considerable importance, sand and gravel being good filtering soils, while chalk is liable to be deeply fissured.

In a recent interesting report, Copeman (Copeman, 1906) has shown that fluorescin solution could be conveyed for at least half a mile in the subsoil water in a soil consisting of deeply fissured chalk. It does not, of course, follow that bacteria would be so conveyed, but the experiments are suggestive.

Pfuhl (Pfuhl, 1897) showed that under the conditions of the experiments and in a gravel soil, *Micrococcus prodigiosus* and a certain vibrio, when added to an experimental pit, passed into the subsoil water, and were detected at a distance of 8 metres away, in one and two hours respectively.

Abba (Abba, Orlandi, and Rondelli, 1899) and his co-workers carried out some experiments upon the filtering power of the soil in connection with the Turin water-supply. They watered prepared areas, over or near the subterranean water conduits, with *B. prodigiosus*, and ascertained how soon it gained access to the water. The time taken by this bacterium to reach the water varied with the circumstances of the different experiments: *e.g.*, with the depth of soil—which was generally at least 2 metres—and particularly with the amount of concurrent flooding with water. In

one experiment the organism was washed through in one and a quarter hours, continuing to appear for nineteen hours subsequently. In other experiments the organism did not gain access to the water under seven hours. In the experiment in which no artificial water irrigation was practised, but in which the bacteria were allowed to be washed in by the rain, which fell heavily, *B. prodigiosus* was not recovered at first, although repeated examinations were made for several days, and the experimental plot was exactly over the underground drain. A month after this experiment this organism appeared in the water from the city taps 19 kilometres (11·8 miles) away, after it had rained for the previous eighteen days.

The authors also investigated the soil of the inoculated patches, and after two months they found *B. prodigiosus* present abundantly to a depth of 3 metres. From the fact that the same organism was found two years later in the Turin water-supply, they concluded that it was present and capable of active life after two years in the soil. These experiments are of great interest as illustrating the power of heavy and persistent rain to wash organisms into the subsoil water.

Excretal Indicators in Soil.

From the point of view from which this chapter is written, it is of great importance to consider how far *B. coli*, streptococci, and spores of *B. enteritidis sporogenes* are present in soils from different sources.

Houston (Houston, 1897-1898) examined twenty-one samples of surface-collected soil from various sources—orchard, garden, peaty, sandy, and pasture soils—and found *B. coli*, or organisms of this group, present in only six of them, although as much as $\frac{1}{3}$ gramme was examined in each case. In regard to the six organisms isolated, they produced colonies on gelatine plates resembling those of *B. coli communis*, grew in broth with

uniform turbidity, formed gas in gelatine shake cultures without liquefaction of the gelatine, and produced acidity and clot in milk. Only four out of the six formed indol. These organisms were from different soils—two from virgin soils not manured, two from manured garden soils, and two from pasture land, the one fed with sewage, the other only possibly sewage-contaminated. As regards *B. enteritidis sporogenes*, in six of the nine virgin soils this organism was not found (in the 0·01 gramme examined), in one it was present 100 to 1,000 per gramme, and in the other two present over 1,000, but less than 10,000, per gramme. In the two orchard soils it was absent in one (in 0·01 gramme of soil); but present in the other in amounts equal to 100 to 1,000 per gramme. This bacillus was found in all the seven garden soils, in six being more than 1,000, and in the seventh between 100 and 1,000 per gramme. The remaining three samples were from pasture land, and all showed more than 1,000 spores per gramme.

In two subsequent investigations upon soil washings, Houston (Houston, 1898-1899, *a*, and 1899-1900, *b*) still further extended his results, examining, however, much smaller quantities of soil.

Eight virgin soils showed no *B. coli*, or coli-like organisms, in the amounts examined, which represented from 0·01 to 0·0001 gramme of soil, but *B. coli*, or coli-like organisms, were isolated from four out of the seven highly polluted soils, the amounts of washings used only representing 0·0001 gramme.

From the results of his three years' investigations Houston states that 'they seem to show conclusively that *B. coli* (or its close allies) is not discoverable, or is present in small numbers only, in *pure* soils. Further, they would seem to indicate that *B. coli* is not readily isolated, even from soils polluted with objectionable animal matters, unless, indeed, the contamination is gross in amount and of *recent* sort.'

Houston also investigated the presence of streptococci in these soil washings. In the first investigation a negative result was obtained in the case of seven soils, virgin or *not recently* polluted, and a positive result with three soils, all of which were known to have been subject to *recent* pollution of excremental sort. In the second investigation a negative result as regards the isolation of streptococci was obtained in the case of all the virgin soils, when washings approximately equal to 0·0001 gramme of soil were used, while a positive result was obtained in four out of the seven polluted soils when working with an equally dilute soil washing. As regards spores of *B. enteritidis sporogenes*, the results obtained in the second report were particularly striking. Of the eight virgin soils selected, not one yielded a positive result with the amounts of soil washings examined, roughly representing 0·01 gramme of soil, while, on the other hand, all seven polluted soils contained spores of this bacillus in large numbers.

The findings obtained by the same observer with ten samples of soil from Chichester (Houston, 1899-1900, *c*) show similar results. The samples were all from the surface of untrodden garden ground which had not been manured for a considerable time. In each instance the samples were taken a few yards away from the back premises of houses. In only two cases were organisms resembling *B. coli* found in the amounts of soil examined (0·01 gramme). The characters of these two organisms were: gelatine surface colonies similar to *B. coli;* no liquefaction of gelatine; acidity in milk, but no coagulation; gas formation in gelatine shake cultures in twenty-four hours at 37° C.; uniform turbidity in broth; no indol production. But few observers would now include such organisms, without additional tests, as *B. coli*, and the results show that even with such an elastic definition of this organism, it is relatively absent from ordinary soil. Streptococci were not found in any of these ten soils in the amounts

(0·1 gramme) examined. Spores of *B. enteritidis sporogenes* were present in all ten soils, at least 1,000 spores per gramme (except in one instance, when they were between 100 and 1,000) being present. In four of the samples more than 10,000 per gramme were present. Houston accepts this as evidence of pollution at one time or another, but not necessarily of recent pollution.

Chick (Chick, 1900) examined fifteen samples of moorland soil for *B. coli*, and it was found to be absent in the amounts examined—0·02 to 0·1 gramme. It was also absent in five out of six samples of dust from the laboratory.

In fourteen samples of (manured) cultivated soil the same author (Chick, 1901) found this bacillus only once: in soil from a cultivated garden. The amounts of soil examined were small, however, only 0·006 to 0·04 gramme.

Horrocks (Horrocks, 1903) records that he has never been able to find in virgin soils bacilli which display all the characteristic reactions of true *B. coli communis*.

The writer (Savage, 1905, *b*) investigated tidal mud, and showed that the number of *B. coli* and streptococci diminished nearly uniformly with the distance from sources of pollution.

In all the ten highly polluted mud samples *B. coli* was present in numbers at least 10,000 per gramme, and streptococci in amounts never less than 100, and frequently at least 1,000 or 10,000 per gramme. Of eight samples from sources not subject to any but remote contamination, in one the number of *B. coli* was more than 100, but less than 1,000, per gramme, but in the others it was either 10 to 100, or less than 10, per gramme, while as regards streptococci the results showed 10 or less than 10 per gramme. The samples intermediate to these as regards pollution showed intermediate results as regards *B. coli* and streptococci.

The writer has also made recently a number of direct examinations of surface soil and soil collected at a depth of 1 foot. In the table on p. 59 are shown the results of the examination of fifteen samples of surface soil and seven

No. of Sample.	Class.	Depth.	Particulars of Soil.	"Excretal" *B. coli.**	Coliform Organisms.	*B. enteritidis sporogenes* Spores.	Streptococci.
				Number per Gramme.			
1.	A	Surface	Virgin soil from a wood	Absent in $\frac{1}{5}$ gramme	None found in $\frac{1}{5}$ gramme	Absent in $\frac{1}{10}$ gramme	Absent in $\frac{1}{10}$ gramme
2.		,,	,, ,,	,,	,,	,,	,,
3.	,,	,,	,, ,, Abundant vegetable matter		,,	,,	,,
4.	,,	,,	,, ,,	,,	,,	,,	,,
5.	B	,,	Grass plot in a garden	,,	,,	100 to 1,000	,,
6.		,,	,, hospital grounds	,,	,,	Absent in $\frac{1}{10}$ gramme	,,
7.	,,	,,	A ploughed field	,,		10 to 100	,,
8.	,,		Old pasture land			Absent in $\frac{1}{10}$ gramme	,,
9.	,,	,,	Pasture land near a well		,,	1,000 to 10,000	,,
10.	C	,,	Garden soil, manured 4 months previously	,,	,,	10 to 100	
11.	,,	,,	,, ,, 2 ,, ,,	Not isolated in $\frac{1}{5}$ gramme	In 0·1 gramme	,,	,,
12.	,,		,, ,, $3\frac{1}{2}$,, ,,	Absent in $\frac{1}{5}$ gramme	Not found in $\frac{1}{5}$ gramme	,,	,,
13.	,,	,,	Cultivated field ,, 13 ,, ,,	10 to 100	,,	,,	,,
14.	,,	,,	,, ,, manured 13 months previously, another part of same field ...	100 to 1,000	,,	100 to 1,000	,,
15.	,,	,,	Garden soil manured 2 weeks previously	100 to 1,000	,,	10 to 100	100 to 1,000
16.	B	1 foot	Same as No. 5	Absent in $\frac{1}{5}$ gramme	,,	10 to 100	Absent in $\frac{1}{10}$ gramme
17.	,,	,,	,, ,, 6	,,	,,	Absent in $\frac{1}{10}$ gramme	
18.	,,	,,	,, 7	,,	,,	10 to 100	
19.	C	,,	,, 10	10 to 100	,,		,,
20.	,,	,,	,, 11	,,	,,	,,	
21.	,,	,,	,, 12	,,	,,		,,
22.	,,	,,	,, ,, 15	100 to 1,000	,,		,,

* For characters of 'excretal' *B. coli*, see p. 226.

samples collected at a depth of 1 foot, by means of a sterile Fraenkel borer. They were taken from soils the previous history of which was well known, and the methods of examination were identical throughout.

The soils are divided into three classes:

(A) Virgin soil samples.

(B) Soils not recently manured—*i.e.*, not within one and a half years.

(C) Soils manured within one and a half years.

The absence of *B. coli* from all the samples of classes A and B is of great significance, while this organism was present in most of the class C samples. The absence of streptococci from all the samples, except one manured within two weeks, illustrates how rapidly these organisms may die out in soil. The *B. enteritidis sporogenes* results are less uniform, showing that the spores may persist for long periods in soil, but in pure virgin soil they were uniformly absent. From a comparison between the surface samples and those taken from the same soil at a depth of 1 foot, and as nearly as possible from the same place, we may conclude that the bacterial content of the soil 1 foot deep is not materially different from the content on the surface.

From these different investigations it is a logical deduction that *B. coli* and streptococci die out fairly readily in soil; but this question, one very important to the water bacteriologist, has been made the subject of direct inquiry.

Houston (Houston, 1900-1901, *a*, and 1901-1902, *a*) investigated the fate of certain sewage organisms—*B. coli*, streptococci, and spores of *B. enteritidis sporogenes* when sown broadcast on soil. The main object of the investigation was to ascertain whether in the surface layers of soil, after it had been inoculated with sewage, these special sewage organisms retained their vitality for any considerable length of time. The first report deals with the inoculation both of soil *in rure* and of soil *in urbe*. For the first a plot of soil of rather poor quality, and not rich in organic matter,

was levelled and systematically watered with cesspool sewage, samples for bacterial examination being collected from time to time. For the second about 4 bushels of loamy soil were placed in a specially-constructed wooden box and watered at intervals with London crude sewage, samples being collected before and between the inoculations.

In the second year's experiments a fresh plot of soil *in rure* was used. This consisted of Thanet sand laid down to a depth of 6 metres, enclosed by a frame, the sand lying upon ordinary soil. In this way a soil of great bacterial purity was obtained.

From his two years' elaborate investigations on these treated soils, Houston draws the following conclusions:

'The addition of sewage to an ordinary garden soil *does not* seemingly lead to a *marked nor, indeed, to other than temporary increase of the sewage microbes in general at the expense of the soil bacteria.* On the contrary, the hardier soil bacteria seem gradually to oust the more delicate sewage microbes in the struggle for existence.

'But the addition of sewage to a *virgin sandy soil* leads to an *enormous increase in the total number of microbes* as compared with the number present in the soil antecedent to the inoculation process. And although *the numbers* rapidly, but not uniformly, *diminish*, the soil *does not*, within a period of *some months, revert to its original state.*

'The addition of sewage to a garden soil tends primarily to *increase the ratio of total number of bacteria to spores of aërobic bacteria*, but this alteration is apt to be soon lost.

'The addition of sewage to a soil leads to an increase in the number of *spores of B. enteritidis sporogenes.* The experiments sometimes seemed to indicate a partial disappearance of the spores of this anaërobe from the soil during the period of observation; but, taking the results as a whole, it may be said that any diminution in the number of spores of *B. enteritidis sporogenes* that may have taken place compared with the marked reduction in the abundance of other *but*

non-sporing microbes of excremental sort—*e.g.*, *B. coli* and streptococci—was small in amount.

'The addition of sewage to a soil greatly alters its bacterial composition in respect of *B. coli and allied forms.* But this alteration tends to become less apparent as time gradually elapses. Sometimes the relative disappearance of *B. coli* is rapid, sometimes much slower, and periods of a *seeming* return to vitality are not uncommon. Moreover, the total disappearance of microbes *seemingly akin* to *B. coli* was by no means always established, when the period of scrutiny was extended over weeks and even months. But there can be little doubt that the experiments, as a whole, tend to confirm my previous inferences, namely, that if *B. coli* does not perish in the surface layers of soil, it certainly becomes greatly reduced in numbers; so that its presence in a soil in any number may be taken as affording reasonable grounds for suspecting pollution of *comparatively recent* sort. The relative death of the completely typical races of *B. coli* was much more apparent than that of the less typical forms. But this may in reality mean that the originally completely typical strains of *B. coli* lose some of their positive attributes during their prolonged sojourn in the soil.

'The addition of sewage to a soil may be detected by the presence of streptococci even in a minimal amount of the soil thus polluted. But the disappearance (relatively, if not actually) of the microbes *unquestionably* to be regarded as streptococci seems to be extremely rapid. Nevertheless, the persistence of certain kinds of streptococci or of pseudo-streptococci for long periods in the soil was observed on more than one occasion.'

Chick (Chick, 1901) examined thirty-three samples of road dust, and found that *B. coli* was almost invariably absent in dry samples, but were present in quantity in road puddles and in wet samples of dirt taken during rainy weather; and from direct experiments made with *B. coli* in wet and dry soils she concluded that the drying of the soil

and dust is an important factor in causing the death of this organism.

The writer (Savage, 1905, *b*) kept tidal river mud in tanks under, as far as possible, natural conditions, the mud being covered with at least 1½ gallons of fresh sea water (partially sterilized before addition), changed twice a week, the mud and water being thoroughly mixed after each addition. Three series of experiments were carried out. The number of *B. coli* and streptococci was estimated every week. When the experiments were started, *B. coli* was present in numbers between 100,000 and 1,000,000 per gramme, and streptococci between 1,000 and 10,000 per gramme, of moist mud. A gradually, but not perfectly regular, decline took place week by week in the number of both *B. coli* and streptococci. The results showed that streptococci usually diminished in number and died out much more rapidly than *B. coli*, and are indicators of more recent contamination of tidal mud. Some resistant forms of streptococci were, however, met with. The decline in the number of *B. coli* was at first rapid, but subsequently comparatively slow, this organism being found in 1 gramme of the mud even after the lapse of three months from the initial pollution.

The distribution of these three organisms in soil, and their vitality therein, has been dealt with in some detail, as it involves questions of the utmost importance in relation to the bacteriological examination of water.

The results here recorded are a complete refutation of the statements repeatedly put forward, but quite devoid of foundation, that *B. coli* is an organism present everywhere, and in abundance in surface soil. As regards *B. coli*, they show that this organism is abundantly present in soils which have been quite recently polluted, while it is absent, or relatively absent, in pure soils or those not recently contaminated. Streptococci, when present, appear to be an even more sensitive test of quite recent fouling. As regards spores of *B. enteritidis sporogenes*, while there is a wide differ-

ence as regards the presence of this organism between a virgin soil and a highly polluted one, rather varying results are obtained with other soils, such, for instance, as comprise most pasture land. Taken as a whole, the data available as to the distribution of these spores shows that they indicate pollution, but possibly long antecedent pollution, and their presence should not be taken as giving more reliable information than that.

Pathogenic Organisms in Soil.

As regards the vitality of pathogenic organisms in soil, the only one with which the water bacteriologist is intimately concerned is the typhoid bacillus.

Bacillus Typhosus in Soil.—The vitality of the typhoid bacillus in soil, and the possibility of its saprophytic existence there, present problems of great importance, and intimately concerned with questions of water-supply. If it can be shown that this bacillus can live for prolonged periods in soil, it is easy to understand how it may gain access to water-supplies. More directly related to the bacteriological examination of water is a consideration of the possibility of its growth in soil taking place, but with extensive alteration of character, so that it is no longer to be recognised with certainty as a typhoid bacillus, thus offering an explanation of the fact that this organism is so infrequently isolated from water-supplies.

Karliński (Karliński, 1891) found that typhoid bacilli lived in soil for as long as three months.

Dempster (Dempster, 1894), working with sterilized soils only, found that the all-important factor influencing the duration of life of the typhoid bacillus, and the cholera spirillum, in sand and garden earth, is moisture, and that these soils have otherwise no marked favourable or injurious action upon these organisms. Peat, on the contrary, proved very deadly to both, exerting a direct inimical action. In moist sterile soils the typhoid bacillus was found alive

in white sand on the twenty-third day, in yellow sand and garden earth on the forty-second day, but from peat not later than twenty-four hours. In soils which had been deprived of their moisture the bacilli were only found up to the seventh day.

Robertson (Robertson, 1898) inoculated selected soil areas with typhoid bacilli, and isolated the bacilli after 88 days and after 142 days, but not subsequently; but in soils treated also with weak organic solutions he recovered the bacillus over 300 days after the original inoculation with the typhoid bacillus. The details as to the degree of dilution used when testing the isolated bacilli with the antityphoid serum are not stated, nor are the cultural characters set out in detail. In view of the fact that the conditions necessary for an organism to be regarded as the typhoid bacillus were not at the time of the investigation, and with the then available knowledge, very stringent, the evidence that the bacilli isolated were true typhoid bacilli cannot be accepted as established with certainty.

Sidney Martin (Martin, 1896 to 1901), in a prolonged series of investigations upon typhoid bacilli in soil, obtained the following results: The earlier reports showed that the typhoid bacillus could live and multiply in certain soils—*i.e.*, organically contaminated soils—for long periods, both at 15° C. and 37° C., when these soils were *sterilized*. The favourable soils are those which have been cultivated, and the bacilli were found alive for as long as 456 days. On the other hand, certain virgin soils, mainly sand and peat, were absolutely inimical to the growth of the typhoid bacillus. In the majority of cases, even on the day following the inoculation of these soils with the bacillus, no evidence of its presence could be obtained. In regard to *unsterilized* soils, very different results were obtained. From the experiments made, Martin concluded that when the typhoid bacillus is added to a well-moistened (but not sloppy) cultivated soil it rapidly dies, and is

usually not obtainable in two days after being sown in it, its disappearance appearing to be the more rapid the higher the temperature. In such a moistened soil, at the temperature of 30° to 40° C., there is a rapid growth of the bacteria proper to the soil, and it was found that it is to the antagonism of these soil bacteria that the disappearance of the typhoid bacillus is due. If the cultivated soil is not made very moist when the bacillus is added, this micro-organism can be recovered up to twelve days after it has been added to it, this being the longest time in any of the experiments after which the typhoid bacillus was regained from any unsterilized cultivated soil. As a final conclusion, Martin states that 'the typhoid bacillus has commonly only a short existence in the soil; that it is destroyed by the products of the putrefactive bacteria which exist in most cultivated soils.'

Rullmann (Rullmann, 1901) found typhoid bacilli after 100 days in unsterilized soil, which was receiving washings of various organic fluids. In sterilized soils the bacilli were recovered after as long as sixteen months.

In a more recent investigation the same worker (Rullmann, 1905) found the bacilli alive after one and a half years in sifted soil, and in rubbish ('made soil') when sterilized and kept at laboratory temperatures, but only found them at the first examination (made after six months) in sterile sand. The characters of the typhoid bacilli were not altered by the prolonged saprophytic life, except that there was some diminution in agglutination power, while some abnormal gelatine colonies were met with.

Firth and Horrocks (Firth and Horrocks, 1902) experimentally investigated the question, and arrived at the following results and conclusions:

'1. That there is no evidence to show that the enteric bacillus, when placed in soil, displays any disposition or ability to either increase in numbers, or grow upwards, downwards, or laterally.

'2. That the enteric bacillus can be washed through at least 18 inches of soil by means of water, even when the soil is closely packed down and no fissures or cracks allowed to exist.

'3. That the enteric bacillus is able to assume a vegetative existence in ordinary and sewage-polluted soil, and survive therein for varying periods, amounting in some cases to as much as seventy-four days.

'4. That the presence or absence of organic nutritive material in the soil appears to be a largely negligible factor, since the enteric bacillus can survive in a soil indifferently well, whether it be an organically polluted soil or a virgin soil, and whether it receives dilute sewage or merely rain water.

'5. That an excess or great deficiency of moisture in soils appears to be the dominant factor affecting the chances of survival of the enteric bacillus in, or at least the possibility of recovering it from, soil.

'6. That from fine sand allowed to become dry the enteric bacillus can be recovered on the twenty-fifth day after inoculation.

'7. That from fine sand, kept moist with either rain or dilute sewage, the enteric bacillus cannot be recovered later than the twelfth day after fouling; this inability to recover the organism is due probably not so much to its death as to its being washed down into the deeper sand layers by liquids added.

'8. That in peat the enteric bacillus appears to rapidly die out, as the microbe cannot be recovered from it after the thirteenth day; but this soil is so porous that it is quite possible that the micro-organism was washed down into the deeper parts, and consequently not recoverable from the place of inoculation.

'9. That from ordinary soil kept damp by occasional additions of rain water the enteric bacillus can be recovered up to and on the sixty-seventh day; when kept damp by

additions of dilute raw sewage up to the fifty-third day; and when by dilute sterile sewage up to the seventy-fourth day.

'10. That in a similar soil, after heavy rainfall, the enteric bacillus at once disappears from the surface layers.

'11. That from a similar soil, allowed after inoculation to become so dry as to be readily blown about as dust, the enteric bacillus can be recovered up to and on the twenty-fifth day.

'12. That in a sewage-polluted soil recovered from beneath a broken drain, the enteric bacillus is able to survive up to the sixty-fifth day.

'13. That the enteric bacillus is able to survive in surface soil an exposure to 122 hours of direct sunshine, extending over a period of twenty-one consecutive days.'

Pfuhl (Pfuhl, 1902) in his investigations found that typhoid bacilli lived in moist garden soil for eighty-eight days, and were found in dry sand after twenty-eight days, but not later.

The writer (Savage, 1905, *b*) found that the typhoid bacillus readily lived in sterile river mud, at first an actual increase in numbers taking place, followed by a decrease, so that at the end of seven weeks the bacilli were about as numerous as when the mud was first inoculated. In highly polluted unsterilized mud from a tidal river inoculated with the typhoid bacillus and treated twice a week with fresh sea water, this organism could be fairly readily isolated after two weeks, but after that time, in three of the four experiments made, they could not be found, although in the remaining experiment they survived for five weeks.

These results show considerable discrepancies. They show clearly, however, that under favourable conditions the typhoid bacillus will survive for a considerable period in soil, and that the factors influencing its vitality are many and varied, the antagonism of other microbes and the physical conditions of moisture and temperature being the

most important. There is no evidence available to show that the typhoid bacillus can, under the influence of saprophytic surroundings, alter its characters in any marked degree or beyond a temporary alteration.

How long the typhoid bacillus can remain alive in excreta has not been definitely ascertained.

Gärtner (Gärtner, 1898) found that this bacillus lived in excrement always for three days, and on one occasion for ten days.

Levy and Kayser (Levy and Kayser, 1903) claim to have isolated the bacillus after five and a half months from the fæces of a typhoid fever patient, which were placed for five winter months in a pit and then spread on a clay soil for fifteen days. The isolated organism was readily agglutinated by a highly dilute anti-typhoid serum, but Pfeiffer's test was apparently not carried out.

Recently Almquist (Almquist, 1906) has shown that both the typhoid bacillus and the cholera vibrio grew luxuriantly in sterilized manure, and that their virulence remained unaltered for several weeks.

From what is known of the antagonism of other bacilli to *B. typhosus*, it is hardly likely that under ordinary circumstances the latter bacillus survives for any length of time.

CHAPTER VI

Bacillus Coli and Allied Organisms

MODERN bacteriological research is continually demonstrating that while any individual bacterium may have well-defined and apparently permanent characters, yet it is not possible to sharply define one species from all others, for all gradations are met with between organisms which are readily defined from one another when considered individually. We have to class bacteria into groups, and to consider the characters of the group as a whole, and of variations within the group.

Of such modern bacteriological tendencies the above group is a very good exemplification. We have not, for example, to consider two individual organisms—*Bacillus coli communis* and *Bacillus typhosus*—and to discuss the differences between these only, but we have these two organisms separated sharply from one another by chemical and morphological characters, yet connected by an apparently interminable number of intermediate forms.

Durham (Durham, 1898) divided the whole *Coli-typhosus* group into three divisions, of which *B. coli*, *B. enteritidis*, and *B. typhosus* may be taken as types.

The characters of this group are not well defined, but morphologically they are all rods with rounded ends, without spores, not stained by Gram's method, with mode of growth aërobic and facultatively anaërobic. To these characters must be added that they are probably all motile, or capable

of exhibiting motility under favourable conditions, that they all grow on gelatine and do not liquefy it (not universally accepted).

Durham's three divisions are :

1. *The Eberth or Typhoid Group*, including the typhoid bacillus and its near allies.

2. *The Gärtner or Hog Cholera Group*, including *B. enteritidis* and its near allies.

3. *The Escherich or B. coli Group*, including *B. coli communis* and its near allies.

The typhoid group is considered in a separate chapter.

The Gärtner or Hog Cholera Group.

The organisms of this group possess characters which, in general, readily separate them from the other two.

This group is one of very great interest and importance, but from the point of view of the bacteriology of water it is of quite secondary significance.

The bacilli included are actively motile, grow on gelatine slope as a translucent growth and without liquefaction, ferment glucose, dulcite, lævulose, and mannite, but not lactose, saccharose, or starch. Maltose is fermented by *B. enteritidis* and by at least most of the group.

In litmus-milk they all (except the Paratyphoid A Group, which is said to produce permanent acidity) produce a slight amount of acid, with subsequent production of distinct alkalinity, the time taken to produce definite alkalinity varying with the different organisms. Indol production is variable—usually nil or slight. The group includes a number of sub-groups, the most important being :

(*a*) The Hog Cholera Sub-group, including the bacillus of hog cholera.

(*b*) The Gärtner Sub-group, including *B. enteritidis* and many of the meat-poisoning bacteria.

(*c*) Paratyphoid A Sub-group.

(*d*) Paratyphoid B Sub-group.

[Sub-groups (*c*) and (*d*) include the organisms found in paratyphoid fever, the majority belonging to the Paratyphoid B Sub-group.]

(*e*) The Psittacosis Sub-group, including *B. psittacosis*, isolated from a disease of parrots.

The distinctions between these sub-groups are almost entirely based upon their agglutination properties.

The essential distinguishing characters of this group are that they ferment glucose, but not lactose, and that they produce alkalinity, after transient acid production, in litmus-milk.

How far these bacteria are natural inhabitants of the human or animal intestine has not as yet been determined with certainty. The writer specially examined the excreta of two cows, one horse, and one pig, and scrapings of the intestines of four cows and eight pigs, for organisms of this group (between December, 1904, and March, 1905). In only four cases, all from pigs, were members of this group met with, and in only one in marked abundance.

Morgan (Morgan, 1905) isolated from the excreta and mucous membrane of pigs, bullocks, sheep, etc., twenty-one cultures of bacilli of the enteritidis type and ten of paratyphoid A.

Although, therefore, they are sometimes found, there can be little doubt that these organisms are but rarely present, or present in extremely small numbers only, in ordinary healthy human or animal excreta; while they are very rarely met with in water-supplies, although the writer has found bacilli of the *B. Gärtner* type in tidal river mud.

In looking for organisms of this group in water, dulcite media should be employed, as recommended by Morgan, Boycott, and others, since this group ferment dulcite well; and, as Boycott (Boycott, 1906), in a valuable paper on this group, points out, on such media they proliferate and tend to outgrow the non-dulcite fermenters.

The B. Coli Group.

The type bacillus is the *B. coli communis* of Escherich, but the group also includes a large number of allied organisms.

The interclassification of this group is a matter of extreme difficulty, in part because the descriptions of many organisms classed as varieties are very incomplete, and in part on account of the bewildering variety of tests introduced by different bacteriologists, each observer using his own set of tests, while practically no investigators have used all the tests. The results obtained are therefore difficult or impossible to properly compare.

In view of the importance of the subject and the diverse positions taken up by different workers, it is necessary to consider the question of the characters of *B. coli* in some detail.

In addition to such ordinary tests as morphological characters and growth in broth and on agar slope, the following are the more important tests which have been advocated and used from time to time in the chief researches made upon organisms of this group:

Character of the gelatine colonies.

Appearance of growth in gelatine stab and slope cultures.

Growth in litmus-milk.

Production of indol.

Growth upon potato.

Growth in a lactose medium for fermentation.

Growth in a glucose medium for fermentation.

Growth in a saccharose medium for fermentation.

Fermentation of other sugars, alcohols, etc., such as starch, dulcite, mannite, sorbite, raffinose, inulin, salicin, glycerine, maltose, galactose, lævulose.

Estimation of the amount of acid produced in litmus-whey.

Neutral red reaction.
Reduction of nitrates to nitrites.
Growth in Proskauer and Capaldi's medium No. 1.
" " " " No. 2.
Voges and Proskauer's reaction.
A determination of the relative amounts of H and CO_2 produced in the different sugar fermentations.
Specific agglutination properties.
Pathogenicity.

From the point of view of the water bacteriologist, these tests are not all necessary, and it is quite impracticable to carry out all of them in routine work. His aim is to detect the variety, or varieties, of this group which are common in excreta and sewage; and the minimum number of tests which will enable him to do this, and yet exclude all pseudo forms not derived from such sources, is all that he requires.

Theobald Smith (Smith, 1895) clearly defined the characteristics which he considered as true to this species, and these have largely been accepted as the standard characters by American bacteriologists. They are—growth on gelatine in the form of delicate bluish or more opaque whitish expansions with irregular margin; active motility when young surface colonies from gelatine plates are examined in hanging drop; coagulation of milk within a few days; growth upon potato as a brownish-yellow deposit, or merely as a glistening, barely recognisable layer; a distinct indol reaction; fermentation of glucose broth, with acid and gas production [total gas approximately half the volume of the closed arm, consisting of H : CO_2, in the proportion of 2 : 1 approximately]; fermentation of lactose broth in similar fashion; saccharose, fermented or not.

Gordon (Gordon, 1897) examined over 100 coli or coli-like organisms, and by means of their reactions and flagella averages classified them into sixteen varieties.

Boyce and his co-workers (Boyce, MacConkey, Grünbaum, and Hill, 1902) defined the *B. coli communis* group as consisting of organisms which have the following characters: They are non-sporing and non-liquefying; they rarely stain by Gram's method; they produce acid and gas with both glucose and lactose, and may do so with saccharose; they produce acid in milk, and usually also coagulate it; they produce acid and gas in bile-salt glucose broth; they grow well at a temperature of 42° C. These investigators found the production of indol very variable, and the coagulation of milk also somewhat unreliable.

Horrocks (Horrocks, 1903) regards the bacilli having the characters of his Group 1 as the only organisms of the *B. coli* type on which implicit reliance can be placed as indicators of sewage contamination. He suggests that the name *B. coli communis* be restricted to this group, and describes its characters as follows: Thin bluish colonies on the surface of gelatine plates, with irregular margin, gelatine not liquefied; broad thin growth, with an irregular margin, on gelatine stroke; gelatine stab preparations show surface growth like the colonies, and thick white growth along the stab; glucose and lactose, peptone solutions both fermented, with acid and gas production in twenty-four hours; cane-sugar fermented or not; nitrates reduced to nitrites; acid produced in litmus-whey, after twenty-four hours' growth at 37° C., the amount of acid produced being very marked after seven days; production of indol in peptone and salt solution; colour changed to yellow in glucose neutral red shake preparations; milk coagulated within seventy-two hours; coloured growth on potato; growth in Proskauer and Capaldi's media, with acid reaction after twenty-four hours in No. 1 and alkaline reaction in No. 2; diffuse growth in broth with surface pellicle; growth taking place under anaërobic conditions; small, feebly or distinctly motile bacilli, decolourized by Gram.

Horrocks states that the growth on potato and power to

produce indol are liable to variation, while in regard to some of the other tests he agrees that they are of little or no differentiating value. The characters which he regards as of chief importance and freedom from variation are gelatine plate colonies (somewhat variable), thin bluish growth on gelatine slope with absence of wrinkling or liquefaction, fermentation of glucose and lactose with marked acid and gas production, formation of acid in litmus-whey, neutral red test (especially in its negative aspects), coagulation of milk, and growth under anaërobic conditions.

Houston (Houston, 1902-1903, *a*) made a very complete study of 101 *B. coli* isolated from normal stools of healthy persons. Of these, he classes 72 per cent. as completely positive, yielding results as follows : Motile ; ' gas ' in gelatine shake cultures ; acid and gas in both glucose and lactose peptone media ; positive result with neutral red broth ; indol formed ; acid and clot in litmus-milk ; gas, acid, and clot in lactose-peptone milk cultures ; reduction of nitrates ; bright cherry-red colour in Proskauer and Capaldi medium No. I. ; bleached appearance, no definite acidity in Proskauer and Capaldi medium No. II. ; percentage acidity in litmus-whey culture $= 28$ to 46 c.c. $\frac{N}{10}Na_2CO_3$.

Of the 101 organisms, 8 were non-lactose fermenters, or fermented, lactose only slightly, and also did not coagulate milk. Of the remainder, 72 were quite typical as above indicated ; 12 were non-motile, otherwise typical ; 2 produced no indol as their only abnormality ; 2 failed to give the neutral red reaction ; 4 were typical, except that they did not coagulate milk. That is, leaving out motility as a distinguishing character, and including the non-motile as also typical, 84 per cent. were completely positive in their characters.

In his sewage work Houston used a broader basis of classification for his *B. coli*, dealing with coli-like microbes rather than with a narrowly defined *B. coli communis*. His chief tests were growth in broth at 37° C., gelatine shake

preparations, determination of indol production, growth in litmus-milk, and the characters of the gelatine surface colonies.

McWeeney (McWeeney, 1904) gave as the following the characters on the strength of which he would regard a bacillus of coliform type as genuine *B. coli:* Appearance of gelatine colony and non-liquefaction of that medium even after a long time; non-retention of the stain by Gram's method; fermentation of lactose with formation of gas and acid; coagulation of milk within four days at 37° C.; production of yellowish-green fluorescence in neutral red agar shake culture; production of indol in liquid peptone media. As regards motility, he remarked, 'On the motility of individuals, or its absence, I hesitate to lay much stress,' while he saw no advantage in studying the pathogenicity of the isolated strains.

The English Committee appointed to consider the standardization of methods for the bacterioscopic examination of water (Report of English Committee, 1904) defined the characters of the *B. coli* as follows:

'A small, motile, non-sporing bacillus, growing at 37° C. as well as at room temperature. The motility is well observed in a young culture in a fluid glucose medium. It is decolourized by Gram's method of staining. It never liquefies gelatine, and the gelatine cultures should be kept for at least ten days in order to exclude a liquefying bacillus. It forms smooth, thin surface growths and colonies on gelatine, not corrugated, growing well to the bottom of the stab (facultative anaërobe). It produces permanent acidity in milk, which is curdled within seven days at 37° C. It ferments glucose and lactose, with the production both of acid and of gas. The typical *Bacillus coli* must conform to the above description and tests. It generally also forms indol (best obtained in peptone-water cultures), gives a thick yellowish-brown growth on potato (greatly dependent on the character of the potato), some-

times (about 50 per cent.) ferments saccharose, changes neutral red (Grübler's), and reduces nitrates, and half the gas produced by it from glucose is absorbable by KOH; and these tests, if time and opportunity permit, may be performed in addition to the foregoing.'

The American Committee on Standard Methods (Report of American Committee, 1905) states that the test for the presence of *B. coli* in water shall not be considered complete unless the organism is isolated in pure culture and is found to show the following characters: Typical morphology, *i.e.*, a nonsporing bacillus, relatively small and often quite thick; motility, when a young broth or gelatine culture is examined; non-liquefaction of gelatine; fermentation of glucose broth, with the formation of about 50 per cent. of gas, of which about one-third (CO_2) is absorbed by a 2 per cent. solution of sodium hydrate; coagulation of milk, with the production of acid, in forty-eight hours or more at 37° C., either spontaneously or upon boiling; production of indol in peptone solution; reduction of nitrates.

The characters adopted for *B. coli* by French and German workers show variations at least as extensive as those of English investigators, the term *B. coli* in many cases being by no means rigidly defined.

These results and opinions illustrate the very considerable diversity of the tests recommended by different workers and authorities. Dealing comprehensively with the matter, a quite typical *B. coli*, a true *B. coli communis*, would possess all the following characters:

A short bacillus with rounded ends, usually single, staining readily with the ordinary aniline dyes, but decolourized by Gram's method, forming no spores. Motile, and possessing one or more flagella. Grows well under both aërobic and anaërobic conditions.

Broth.—Rapid growth at 37° C., rendering it uniformly turbid.

Agar Slope.—A thick white growth.

Gelatine Surface Colonies.—Visible after twenty-four hours' incubation at 20 to 22° C., and well developed after forty to forty-eight hours. Under a low power of the microscope they appear as flat, colourless, or pale brown colonies, with an irregular outline and a wavy indented margin. Frequently, but not invariably, present over the surface and extending in from the margin, and particularly observable near the periphery, are a number of wavy lines. The gelatine is not liquefied.

Gelatine Slope.—Rapid growth visible within twenty-four hours. Within forty-eight hours seen as a bluish, translucent, smooth growth, which remains unaltered on prolonged (two weeks') incubation.

Litmus-Milk.—Permanent acid formation and coagulation of the milk within a few days.

Indol present when tested for after five to seven days' incubation at 37° C. in peptone water or sugar-free broth.

Litmus - Whey. — A marked amount of acid formed. According to Horrocks (Horrocks, 1901, *a*) it always requires more than 6 per cent. $\frac{N}{10}$ alkali, and usually from 20 to 40 per cent. of $\frac{N}{10}$ alkali is required to neutralize it.

Potato.—A brownish growth ; no gas bubbles.

Glucose Media.—Fermented, with both acid and gas production.

Lactose Media.—Fermented, with both acid and gas production.

Saccharose Media.—Not fermented.

Starch Media.—Not fermented.

Glucose Neutral Red Broth or Agar. — The red colour reduced to yellow, and greenish fluorescence produced.

Nitrate Broth.—Nitrite produced from reduction of the nitrate.

Proskauer and Capaldi's Medium No. 1.—Growth and acid reaction after twenty-four hours at 37° C.

Proskauer and Capaldi's Medium No. 2. — Growth and medium alkaline after twenty-four hours at 37° C.

Voges and Proskauer's Reaction.—Reaction not given.

Reaction to Specific Sera.—It is not agglutinated by highly dilute antityphoid serum.

Pathogenicity.—Highly variable; may or may not be pathogenic to guinea-pigs or mice.

This is a fairly comprehensive list of characters, and it is obviously impossible to carry them all out in routine water examinations. Since the detection and numerical estimation of *B. coli* is by far the most important part of the bacteriological examination of water, it is necessary to discuss in some detail how far this long list of tests can be abbreviated without detriment to the practical information obtained.

The origin of the application of some of these tests to this bacillus will help to simplify matters. Many of the tests applied to *B. coli* were designed to distinguish between this organism and the typhoid bacillus, and are of little value for the identification of *B. coli* itself, or for its differentiation from closely-allied organisms, although excellent for their original purpose. Other and simpler tests are now available to readily distinguish these two organisms.

Thus, Proskauer and Capaldi's two media—No. 1, an asparagin, mannite, magnesium sulphate, sodium chloride, calcium chloride, potassium hydrogen phosphate solution, and No. 2, a peptone mannite solution—are of but limited value for *B. coli* work; they are complicated and quite unnecessary.

Such a large number of organisms grow with a coloured growth on potato that this test is quite useless, although as a differentiating test for the typhoid bacillus it was once considered to be of considerable value. The formation of nitrites from nitrates is a property possessed by all *B. coli*, but it is shared by so many other organisms that its value as a positive test is reduced to a minimum. In the same way the broth and agar slope growths are quite non-characteristic, and the performance of these tests serves no useful purpose. The agglutination reactions are of

value to distinguish the coliform organisms from the typhoid bacillus, but they have not been shown to be of any value for the determination of *B. coli* or any special kind of *B. coli*.

The gas ratio estimation has not been widely employed in this country, although the American results show it to be of some diagnostic value.

Pathogenicity is a highly variable character in the case of *B. coli*, and since the majority of these organisms in fæces are non-pathogenic, the advantage of testing for it as a routine measure is not obvious. The Voges and Proskauer reaction is of diagnostic value, since it is given by at least many organisms of the *B. lactis aerogenes* group, and possibly by other allied organisms, which are indicated by other tests; therefore as a routine test it is hardly necessary.

The gelatine surface colonies are as described above in the majority of cases, but are liable to some variation. It is not necessary as a routine practice to make gelatine plates, since they supply very little valuable information in addition to that yielded by the surface gelatine slope growth.

The gelatine slope characters are important and stable, although sometimes the growth is somewhat whiter and more opaque than above described. Liquefaction of the gelatine is now generally accepted as at once excluding from the *B. coli* group. Horrocks (Horrocks, 1903), under the name *B. sulcatus gasoformans*, has decribed an organism occasionally met with by him in surface waters, which closely resembles *B. coli*, the essential difference being that this bacillus grown on gelatine slope shows after about five days a corrugated or folded appearance, the same characteristic being shown in the gelatine plates.

In regard to the amount of acid produced in litmus-whey, Durham, Horrocks, and Houston all regard it as of considerable value. Houston found that 96 per cent. of the coli-like microbes isolated by him from fæces required from

28 to 46 per cent. $\frac{N}{10}$ Na_2CO_3 to neutralize the acidity. This estimation is a valuable one, and one apparently not subject to variation.

These considerations enable the essential, but sufficient, characters of a true *B. coli communis* to be reduced to the following:

A short bacillus with rounded ends, no spores, motile.
Decolourized by Gram's method.
Characteristic growth on gelatine slope.
Acid production and coagulation in litmus-milk.
Production of indol.
Production of a considerable quantity of acid in litmus-whey.
Fermentation, with production of both gas and acid, in glucose and lactose media.
No fermentation of starch or saccharose.
A positive neutral red reaction.

But variations from this typical form are met with. The most important is that all the above characters are present, except that saccharose is also fermented. This is not the *B. coli communis* of Escherich, since that organism does not ferment this sugar; but otherwise it is identical. Houston found that 70 per cent. of *B. coli* from human fæces either did not ferment saccharose at all or fermented it only feebly; in sewage he states that the number of saccharose-fermenting *B. coli* is extremely great. All workers class both groups as of equal value as excretal indicators, although the name *B. coli communis* can only be strictly applied to Escherich's organism. This saccharose-fermenting variety of *B. coli* has been called by Durham *Bacillus coli communior*, shortened by Ford into *Bacillus communior*.

Such a distinction is, however, quite unnecessary from the point of view of the bacteriology of water, and, indeed, by the use of further substances, such as dulcite and raffinose, it is possible to further subdivide otherwise quite

typical and apparently identical *B. coli*. For practical purposes, it is unnecessary to maintain this distinction, and organisms with all the above characters, whether they ferment saccharose, dulcite, etc., or not, can all be spoken of as *B. coli*.

But apart from the action on these bodies, numerous other deviations from the typical *B. coli* are met with. Such variations fall naturally into two divisions:

1. Variations due to loss or absence of certain positive attributes.

2. Variations due to the addition of certain characters not possessed by *B. coli*, as defined above.

Thus under the first group we find organisms which are quite typical *B. coli*—the word 'typical' being conveniently used as signifying that the characters are as described above —except that they exhibit no motility, or that they produce no indol, give no neutral red reaction, or do not coagulate milk. Absence of more than one attribute—for example, failure to coagulate milk and to produce indol—is also fairly often met with.

In fact, successive loss of diagnostic characters may be met with, until the organism found differs very widely from a *B. coli* as above described.

How far these variants can be called *B. coli*, or, what is of greater practical value, how far they can be used as indicators of equal significance, is a matter of very great importance in practical work, and is fully considered in Chapter X.

In the second group even more marked differences are met with, since added characters are possessed. Under this head would be included organisms typical, except that they slowly liquefy gelatine. Other examples are the production of pigment, fermentation of starch, very excessive gas production, etc.

It is very rare to find any organism which differs from *B. coli* in the possession of one *only* of these added characters. Almost invariably they differ in others respects

also—for example, they frequently fail to coagulate milk or to give an indol reaction.

Obviously, the possession of added and alien properties is a more justifiable ground for removal from the *B. coli* group than mere loss of certain attributes, and, indeed, all the members of this second group of organisms are rejected by almost every bacteriologist as not *B. coli* at all.

Of these allied organisms a considerable number have been named and described by different workers. Unfortunately the characters of many of these bacteria were incompletely described in the original communication, and the same name has subsequently been employed to describe organisms which are certainly not identical. So much is this the case that it is doubtful if the retention of at least the majority of these names is of any service to water bacteriology, and certainly it is useless unless their characters are carefully defined and generally accepted.

Of such organisms the most important are *Bacterium lactis aerogenes*, *B. cloacæ*, *B. acidi lactici*, *B. neapolitanus*, *B. capsulatus* of Pfeiffer, and *B. cavicida.*

Of these, only *Bacterium lactis aerogenes* and *B. cloacæ* are, as far as present knowledge is available, worth further consideration from the point of view from which this book is written. Probably neither are members of the coli group, but they are conveniently considered here.

Bacterium Lactis Aerogenes.

Obtained by Escherich in 1885 from the contents of the small intestine of children and animals fed upon milk, in smaller numbers from the fæces of milk-fed children, and in one instance from uncooked cow's milk.

Under this title two quite distinct bacteria, or groups of bacteria, have been described. One is a bacterium with the definite characters described below, and readily recognisable; the other has been described as identical with *B. coli*, except that it is non-motile.

Dealing with the latter first, Theobald Smith (Smith, 1895) describes one variety as a *B. coli*, except for its want of motility and differing morphology, describing it as shorter and thicker than *B. coli*. Thresh (Thresh, 1904, p. 364) takes the same view, including in the *B. lactis aerogenes* group all the organisms which corresponded with the *B. coli* group in all respects save that of motility.

Lehmann and Neumann (Lehmann and Neumann, 1904, p. 291) consider that this organism is identical with the *Bacterium acidi lactici* of Hüppe, quoting Kruse, Würtz, and Leudet in support of this view, and stating that it is only distinguishable from *B. coli* by its want of motility.

Such an organism is not the *Bacterium lactis aerogenes* of Escherich. Undoubtedly such organisms are met with, but in the writer's experience they are nothing more than *B. coli* which have, under the influence of their environment, lost their power of exhibiting motility when examined by the arbitrary methods selected, and usually by suitable treatment they can be made subsequently to show well-marked motility.

The true *Bacterium lactis aerogenes* group is a very definite and easily recognised one, and its special characters are described by most writers on the subject (Durham, 1900-1901; Sternberg, 1901, p. 536; Horrocks, 1901, *a*, p. 95; Chester, 1901, p. 128, etc.).

The characters of *Bacterium lactis aerogenes*, better called *Bacterium aerogenes* (Migula), are clearly given by Ford (Ford, 1903). He describes its essential characters as follows: Short thick bacteria usually, but longer forms are met with. Milk cultures show the development of a capsule, the presence of which contributes to the peculiar thick form of the micro-organism. Ford points out that the morphology of this bacterium is always characteristic, and is of prime importance in its identification. No spores. No motility to be demonstrated at any time.

Agar Slant.—Abundant thick white glistening growth.

Broth.—Great turbidity, abundant sediment, and usually scum formation.

Gelatine Stab.—Thick growth along line of inoculation and spreading over the surface of the gelatine. No liquefaction.

Gelatine Surface Colonies.—Thick opaque porcelain-white.

Potato.—Thick yellowish-white or yellowish-brown growth, with peculiar wart-like elevations along the edges and upon the surface.

Glucose Broth.—Acid and much gas production.

Lactose Broth.—Acid and gas production.

Saccharose Broth.—Acid and gas production.

Indol.—Usually not produced, but occasionally typical cultures give a positive reaction.

Litmus-Milk.—Acid production and coagulation of the milk.

To this description may be added that the growth on gelatine slope is much thicker and whiter than that of *B. coli.* Also, according to Durham (Durham, 1900-1901) and MacConkey (MacConkey, 1905), the Voges and Proskauer reaction is given, while starch and inulin, one or both, are generally fermented, with acid and gas production.

Ford describes, under the name *Bacterium duodenale*, an identical organism, differing only in not fermenting saccharose.

No doubt, just as in the case of *B. coli*, there are a number of organisms included in the *Bacterium aerogenes* group which have the essential group characters in common, but differ in regard to their powers of fermenting saccharose, dulcite, inulin, and other bodies, and in their power to form indol, etc.

The Voges and Proskauer test (Voges and Proskauer, 1898) consists in adding caustic potash solution to the glucose fermentation tube after growth and gas formation have taken place. The tube is allowed to stand for twenty-four hours or longer at room temperature, and a positive result is shown by the development of a red fluorescent

coloration, somewhat similar to that of a dilute alcoholic solution of eosin.

Distribution and Significance.—The extent to which this organism is said to be present in excreta varies greatly according to different observers. Ford, in his investigations upon intestinal bacteria, found nearly 200 out of 700 organisms to be cultures of typical *Bacterium aerogenes*, and includes it as one of the most frequently encountered and most widely distributed of all the intestinal bacteria, and at one time or another met with in every region of the bowel. He isolated it from thirty-one out of fifty human cadavers examined. He found it to be most frequent in the stomach and duodenum, but also in the cæcum and rectum, where it dwells side by side with *B. coli.*

Booker, in his work on the excreta of infantile diarrhœa cases, found it to be one of the organisms most frequently present.

Heinick (Heinick, 1903) found it to be very numerous in the intestinal canal of pigs, being nearly as abundant as *B. coli.*

Other observers have also found this bacterium common in the dejecta. On the other hand, MacConkey (MacConkey, 1905) isolated 241 lactose-fermenting organisms from 23 samples of human fæces, and only 4 of these were shown to definitely belong to the lactis aerogenes type; while out of 99 bacteria from the horse, cow, and calf (11 samples in all), none were *Bacterium lactis aerogenes.* Altogether, out of 625 lactose-fermenting bacteria, MacConkey only identified 4 as belonging to this group.

This wide discrepancy in results is remarkable, but is, no doubt, largely due to the fact that quite different organisms have been included under the term *Bacterium lactis aerogenes.* Obviously, those who consider this organism to be simply a non-motile *B. coli* will obtain very different results as regards its distribution from those who give it the characters described above.

As regards its significance when found in water, nothing definite can be said, with our present lack of knowledge, of its distribution. It certainly is not abundant in water-supplies, but it is met with from time to time. It is probably an indicator of excretal pollution, since, as far as we know, it is an excretal bacillus, and is not found in unpolluted sources; but more definite information is required.

B. Cloacæ.

First isolated by Jordan (Jordan, 1890) from sewage, and described as one of the commonest organisms therein. He gave its essential characters as:—a short oval bacillus with rounded ends; no spores; very motile. Gelatine surface colonies thin, bluish, and translucent; rapid liquefaction; moist porcelain-white growth on agar slope; yellowish-white growth on potato; in milk acid production and coagulation; in broth turbidity, generally with scum formation; nitrates reduced to nitrites.

In a later paper, Jordan (Jordan, 1903), in classifying the kinds of bacteria found in river water, groups the organisms under the head of *B. cloacæ* as characterized by an 'inverted gas formula'—that is to say, an excess of CO_2 over H in the dextrose fermentation tube. 'Sucrose (*i.e.*, saccharose) is fermented by all cultures, and lactose, although often very slowly, by the majority (14 out of 21 cultures). Considerable variation is shown in the action upon gelatine—2 cultures liquefying rapidly, 14 slowly, 4 only after thirty to forty days, and 1 not at all. All are actively motile. Milk is curdled by all cultures, with acid reaction, and the casein is dissolved by 13 cultures. Only 8 cultures out of 21 produced indol. Blood-serum was liquefied by 5 cultures out of 16 tested.'

Jordan places this group with the proteus forms, as more allied to them in morphology, habitat, action upon sugars, and proteolytic power. He remarks (*ibid.*, p. 10): 'Lactose

fermentation with gas production is absent in the Proteus group, and is either absent or feeble in most members of the *B. cloacæ* group.'

Ford (Ford, 1903) describes this organism as fermenting dextrose, saccharose, and lactose, slowly but completely liquefying blood-serum, usually producing indol, and growing in milk with a slow development of acidity and eventual coagulation. As regards the distribution of organisms of this group, Jordan found *B. cloacæ* a common organism in sewage, and not infrequent in river water. Ford isolated it from the alimentary canal of nine cadavers out of fifty examined. He also found a non-saccharose variety on five occasions.

MacConkey (*ibid.*), in 23 samples of fæces, from which 241 lactose-fermenting organisms were isolated, did not find any bacilli of this group. He occasionally found it in animal fæces. Out of 625 lactose-fermenting bacilli isolated by this observer, 19 were identified as *B. cloacæ*. He remarks that organisms of this group give the Voges and Proskauer reaction.

At present we have but little information as to the distribution of this organism in Nature, so that to what extent its presence indicates sewage or excretal pollution is unknown.

CHAPTER VII

The Eberth or Typhoid Group

This group includes *B. typhosus* and allied organisms.

B. Typhosus.

The following are the essential characters of this organism:

A rod-shaped bacillus with rounded ends, usually about 3 μ long. No spores. Actively motile. Flagella on an average eight to twelve, disposed all round the bacillus. Stained by the ordinary aniline dyes, but not by Gram's method.

Killed by exposure to a temperature of 65° C. for ten minutes. 140°F

Aërobic and facultatively anaërobic.

Agar Slope.—Thin grayish-white, smooth growth, well marked after twenty to twenty-four hours' incubation at 37° C.

Gelatine Slope.—Translucent growth. No liquefaction of the gelatine.

Gelatine Surface Colonies.—Thin bluish, translucent, with an irregular margin, and usually marmorated (wavy lines over the surface). Growth slow, so that these characters are rarely developed under three days' growth at 20° to 22° C. No liquefaction of the gelatine.

Broth.—Uniform turbidity without scum.

Potato (with an acid reaction).—Moist, shining, almost invisible growth. If the potato has an alkaline reaction the growth may be yellowish.

Litmus-Milk.—Grows well, producing a small amount of acid, but never causing coagulation.

Usually the acidity produced in milk is permanent, but occasionally undoubted typhoid bacilli, after an initial acidity production, produce alkali, and the milk becomes blue again. Horton-Smith (Horton-Smith, 1900) tested twenty strains, and remarks that at the end of a month the reaction was nearly always acid, but that occasionally the reaction was faintly alkaline. Of eight races tested in litmus-milk by the writer in the early part of 1905, one of them produced alkalinity (after initial acidity) within two weeks; the other seven remained acid.

Boycott (Boycott, 1906), describing the four typhoid strains used in his experiments, records that all produced an initial acidity in litmus-milk, but that two in nine days and one in fifteen days were markedly alkaline, while the fourth did not produce definite alkali until after a month's incubation.

Litmus-Whey.—A slight development of acid takes place. According to Horrocks, after seven days' incubation at 37° C. the typhoid bacillus never requires more than 6 per cent. of $\frac{N}{10}$ alkali to neutralize it.

Indol Production.—Best tested for in peptone-salt solution after seven days' incubation at 37° C. As a rule no indol is produced, but undoubted typhoid bacilli may produce a little indol.

Proskauer and Capaldi's Medium No. I.—No change when grown for twenty-four hours at 37° C.

Proskauer and Capaldi's Medium No. II.—Grows well, and produces marked acidity after twenty-four hours at 37° C.

Neutral Red Glucose Broth (or Agar Shake).—No colour change.

Lactose Broth or Peptone Solution.—No acid or gas produced.

Glucose Broth or Peptone Solution.—A little acid, but no gas, produced.

Saccharose Broth or Peptone Solution.—No acid or gas produced.

With regard to its action upon other sugars and alcohols, the writer, in 1905, tested thirteen different strains of typhoid bacilli obtained, by the kindness of their directors, from several different London laboratories. They all fermented glucose, mannite, maltose, galactose, and lævulose, with acid, but no gas, production, while none of them had any visible action upon lactose, saccharose, or raffinose. No differences in these respects were made out between the different strains examined. The four strains used by Boycott (*ibid.*) gave similar reactions; in addition, they did not ferment dulcite, salicin, or inulin, but produced acid from sorbite.

Reaction with Antityphoid Serum.—The typhoid bacillus is readily agglutinated by highly diluted antityphoid serum. In view of the fact that some cases of enteric fever are mixed infections, so that the agglutinins of other bacilli may also be present, and also that such sera frequently will not agglutinate in high dilution, all agglutination testings should be made with the serum of an animal immunized against the typhoid bacillus, and not with the blood of a typhoid fever patient. A powerful antityphoid serum should be used, and one which gives a positive reaction when diluted 1 : 1,000.

Immunization of Animals Test.—In doubtful cases an important means of distinguishing between pseudo-typhoid bacilli and true typhoid bacilli is by means of the immunization of animals. A rabbit immunized by repeated injections of a genuine typhoid bacillus will yield a serum which will agglutinate in high dilution other races of *B. typhosus*, while the typhoid-like bacilli from saprophytic sources are incapable of producing sera which have this power. In immunizing animals against this bacillus it is convenient to start with the injection of killed cultures, followed in a few days by small doses of living bacilli, the number of bacilli

injected being gradually increased until a sufficiently powerful serum is obtained. A few drops of the blood are withdrawn from time to time, and the agglutination power of the serum measured.

Pfeiffer's Test.—This consists in injecting a ten times fatal dose of the bacillus, together with a small quantity of serum from an animal highly immunized against the typhoid bacillus, into the peritoneal cavity of a guinea-pig. If the suspected organism is the typhoid bacillus, then the bacilli are converted into granular masses (tested by removal and examination of a little peritoneal fluid after half and after one hour), and the animal does not die, while a control animal injected with the bacillus alone, dies.

Pathogenicity.—Intraperitoneal injection of living cultures usually produces death in guinea-pigs and mice, but different races show considerable variation in virulence. The virulence can be raised by passing the particular strain of typhoid bacillus under investigation through a series of rodents.

At one time, in view of the few distinguishing tests known, it was necessary to discuss in detail the characters which differentiated the typhoid bacillus from *B. coli communis*. At the present day this is unnecessary, since the differences between these two organisms are so marked that confusion cannot possibly arise, and the differential diagnosis has not to be made between these bacilli, but between the typhoid bacillus and certain closely allied organisms which probably belong to the Eberth group.

Houston (Houston, 1898) isolated from the mud of the river Thames four organisms which in many of their characters resembled the typhoid bacillus; all four, however, failed to be agglutinated by antityphoid serum. Horrocks (Horrocks, 1901, *a*, p. 190) isolated two organisms from a polluted water which still more closely resembled the typhoid bacillus, as one of them was agglutinated by antityphoid serum diluted 1 : 80.

Most workers, indeed, who have experimented much with soil, mud, sewage, or water, examining it for the typhoid bacillus, have come across organisms which in many of their characters closely resemble the typhoid bacillus, although careful investigation, particularly agglutination experiments, enables a definite diagnosis to be arrived at.

It is highly probable that there are a large series of saprophytic organisms which, at least as regards their morphological and cultural characters, closely resemble the typhoid bacillus. In view of this probability, it becomes a necessary procedure to apply to all suspected organisms practically the whole of the above tests, and to only accept such an isolated organism as a genuine typhoid bacillus if it conforms to all of them.

Claims have been made in many instances that typhoid bacilli have been isolated from water, soil, etc. For practically the whole of the earlier work such claims cannot be accepted as substantiated, since the existence of these closely allied bacilli was not known, and the tests considered sufficient to satisfy the investigator that the typhoid bacillus was present were few and quite inadequate. In a number of recent cases the tests applied have been much more thorough, and for a few of these the evidence seems sufficient to justify the statement that the typhoid bacillus was isolated.

In a recent paper, Willson (Willson, 1905) summarizes the instances in which *B. typhosus* has been successfully isolated from infected water-supplies. Only those cases are included in which the diagnosis was confirmed both by agglutination tests and by Pfeiffer's reaction. He gives the following:

1. By Lösener, 1895, from the Berlin Waterworks.

2. By Kübler and Neufeld, 1898, from a well on a farm. By direct plating on Elsner's medium. Infection probably through the urine of a typhoid patient.

3. By Fischer and Flatau, 1901, from a well in Schleswig-Holstein. Isolated on carbol-gelatine plates.

4. By Konradi, 1902, from a well in Hungary. Isolated by direct plating on carbol-gelatine.

5. By Jaksch and Rau, 1904. Both from the town water of Prague, and also from the river Moldau. By Hoffmann and Ficker's method, plating out on Drigalski-Conradi medium.

6. By Ströszner in 1904, from a shallow well near Budapest. Method of isolation similar to No. 5.

In no less than three the contamination was supposed to be by urine.

Willson also mentions the following five cases where the isolated organism reacted positively to agglutination tests; but Pfeiffer's reaction was apparently not applied, so that proof of identity cannot be regarded as complete:

(*a*) Wilson and Wesbrook, 1897, from a public water-supply in Minnesota.

(*b*) Hankin, from wells, etc., in India.

(*c*) Genersich, 1899, from public supply at Pécs in Hungary.

(*d*) Tavel, 1902, at Olten.

(*e*) Bonhoff, 1902, from a well near Marburg.

There is another point of view to be considered which bears upon the question of the presence of typhoid bacilli in saprophytic surroundings. It has been contended that the typhoid bacillus in a saprophytic condition may have its characters so modified that when isolated it does not satisfy the above stringent series of tests, although the isolated organism is a true descendant of an undoubted typhoid bacillus.

Thus, for example, Fodor and Rigler (Fodor and Rigler, 1898) contended that a saprophytic environment may cause typhoid bacilli to lose their powers of agglutination, and also the power to produce, by injection into animals, a serum with agglutination properties.

Rémy (Rémy, 1900) studied the antagonism of *B. coli* and *B. typhosus* in neutral and also in slightly acid peptone water (peptone, 3 per cent.; sodium chloride, 0·5 per cent.) kept at room temperatures. From the mixtures of these two organisms in these media, inoculations were made into his special medium, and plates were prepared He concluded that the life in common can modify profoundly the properties of the two organisms, the typhoid bacillus losing its sensibility to agglutination, and the colon bacillus being deprived of its specific characters. When the two organisms were grown separately in the neutral peptone solution, he found them still alive after four months, and with their specific reactions maintained.

On the other hand, Horrocks (Horrocks, 1899) grew the typhoid bacillus in sewage, and found no alteration in its essential characters or in its power of being agglutinated, the only alteration being a diminished resistance to carbolic acid and some modification in the appearance of the gelatine colonies. Jordan (Jordan, 1905) has recently carried out experiments in the same direction, and concludes that the typhoid bacillus may be isolated without special difficulty after association with *B. coli* in tap water and sewage for as long as thirty-four days, and that under these conditions the strains of *B. typhosus* experimented upon retained their property of agglutinability absolutely intact.

The majority of workers who have studied the vitality or the typhoid bacillus under saprophytic conditions also record no alteration in the characters of this bacillus from the action of the environment. For particulars of such investigations see Chapters IV. and V.

On the whole, the actual evidence that the typhoid bacillus may lose some of its characteristic properties by a prolonged saprophytic existence is weak and unconvincing, and will require much more experimental support before it can be seriously considered.

With regard to the relative value of the different characters of this bacillus, no single one of them is specific, with the exception, perhaps, of Pfeiffer's test. Organisms certainly not the typhoid bacillus may give almost all the cultural tests, while typhoid bacilli will sometimes yield traces of indol, and give a slightly coloured growth on potato.

The reaction with antityphoid serum is also not absolutely specific and characteristic. Even some normal sera may agglutinate this bacillus when very low dilutions are employed, while organisms of the Gärtner and colon groups may be agglutinated by antityphoid serum even considerably diluted, as shown by Durham and numerous other observers.

Organisms which are not the typhoid bacillus, but which agglutinate typhoid sera, have been found by several investigators in water-supplies.

Lorrain Smith and Tennant (Smith and Tennant, 1899) found that in the water-supply suspected of being related to the epidemic of typhoid fever in Belfast in 1898, many of the races of *B. coli* isolated, when tested with the serum of typhoid fever patients, showed a well-marked reaction.

Sternberg (Sternberg, 1900) isolated, from waters in the neighbourhood of Vienna, five organisms of the colon group, all of which were very sensitive to typhoid serum, three being quite as sensitive as the typhoid bacillus, and one more so. The *B. perturbans* isolated by Klotz (Klotz, 1904) from two separate waters, one a tap water and the other a polluted river water, is an organism of the colon group which showed marked agglutinative properties, giving a positive reaction with both human typhoid serum and the serum of a rabbit immunized against the typhoid bacillus, in dilutions of over 1 in 1,000.

These considerations emphasize the need for employing *all* available tests, and the only practical procedure at the

present day is to accept as *B. typhosus* only bacilli which conform to all of them.

Viability under Saprophytic Conditions.

The viability under saprophytic conditions of this organism has already been considered as far as sewage and soil are concerned (see Chapters IV. and V.). The longevity of the typhoid bacillus in water may be conveniently considered here.

A large number of experiments have been carried out by numerous observers to see how long the typhoid bacillus will survive in water, both sterilized and unsterilized, but the conditions of experimentation have been so varied that very different results have been obtained. The Franklands in their valuable text-book (Frankland, G. and P., 1894) give a tabulated list of the chief investigations carried out up to 1894, which show very variable results obtained.

Frankland (Frankland, 1895) investigated the vitality of the typhoid bacillus in Thames water, Loch Katrine water, and in water from a deep well in the chalk. In unsterilized Thames water it could not be found after nine days, but in the same water sterilized it was found for the forty-eight days of the experiment. In unsterilized Loch Katrine water it was found alive after seventeen and nineteen days at winter temperature (6° to 8° C.), but had died out when kept at a summer temperature of 19° C. In the same water sterilized, it was found alive in two different experiments, thirty-nine and fifty-one days after its introduction. In the deep-well water it lived for thirty-three days.

Klein (Klein, 1894-1895, *b*) found the typhoid bacillus alive after long periods in sterilized tap water from the London Water Companies. In unsterilized tap water in glass flasks, kept at room temperature, the typhoid bacillus was isolated (in 0·1 c.c.) from each of the three kinds of water used, eighteen days from the start of the experiment. Thirty-six days after the inoculation with the

bacillus it was found in one only of the three waters, and after forty-two days it was not found in any of them. In the sterilized water Klein found the bacillus alive for over eighty-five days.

The results of these and numerous other earlier experiments show that the typhoid bacillus will usually live much longer in sterilized than in unsterilized water.

In the former they may persist for two months, or even longer, while in the latter their vitality is much less, so that they usually disappear within a few weeks. The results of many of these earlier investigations, as regards unsterilized water, must be received with caution, since the diagnostic characters of the typhoid bacillus at the time they were carried out were not sharply defined or sufficient, and some of the organisms recovered were possibly not typhoid bacilli, but related organisms not to be differentiated by the tests then available. At the same time the methods for the detection of this organism were highly imperfect and unreliable, so that typhoid bacilli actually present may have been overlooked.

As Jordan, Russell, and Zeit (*infra*) point out, 'from these experiments substantial accord has resulted on two points: first, that typhoid bacilli die out more rapidly in unsterilized water than in the same water sterilized by heat; second, that when typhoid bacilli are introduced into unsterilized water containing little organic matter, their longevity is more prolonged than in water charged with considerable organic matter. As regards application of results hitherto obtained to particular cases, the available evidence appears to indicate that the conditions of laboratory experiments are so far removed from the conditions found in nature as to afford conclusions of doubtful value.'

Recently a very careful investigation has been carried out by Jordan, Russell, and Zeit (Jordan, Russell, and Zeit, 1904) upon the longevity of the typhoid bacillus in water. Three independent series of experiments were carried out,

Zeit working with Lake Michigan and Chicago River water, Jordan with the Chicago Drainage Canal water, and Russell with the Illinois River water.

Freshly isolated typhoid cultures were used. Most of the modern and more sensitive methods for determining the presence of the typhoid bacillus were used, such as the various enrichment and precipitation methods, the use of the solid medium of Drigalski-Conradi, bile-salt agar, glucose-litmus agar, and the special medium of Hiss. A novel feature of the investigation was that the majority of the experiments were conducted, not in the usual glass receptacles, but in sacs of either celloidin or parchment, inoculated with typhoid bacilli, and suspended in the flowing river water. In this way the conditions were made to approximate much more closely to those met with in nature than the usual experiments of this kind, since these sacs allowed dialyzable substances to pass in and out.

In the comparatively pure Lake Michigan tap water, with only 100 to 2,000 organisms per c.c., the added typhoid bacilli were found alive in the tap water contained in the glass vessels up to seven days, but never later. In the parchment and celloidin sacs suspended in flowing tap water they were found up to seven days, and in one instance eight days.

When the water in the glass vessel and sacs was previously sterilized the typhoid bacilli were found up to twenty-five days (glass vessel), fifteen days (parchment sac), and fifteen days (celloidin sac) respectively, but not later.

In the impure Chicago River water, with a germ content of about 1,500,000 per c.c., they were found alive up to three to four days as a limit.

In the Chicago Drainage Canal water (really a diluted sewage with 4,000 to 6,000 colon bacilli and 1,000 streptococci per c.c.), celloidin and parchment sacs suspended in the flowing water were alone used. Typhoid bacilli in numbers

varying from about 600 to 800,000 were added to the unsterilized Drainage Canal water contained in these sacs. In twenty-seven out of the twenty-eight separate experiments made, no typhoid bacilli were found in plates made from any of the sacs later than two days after inoculation, the decrease in numbers being most marked between twenty-four and forty-eight hours. Three colonies of typhoid bacilli were found on the tenth day after inoculation in one experiment. They were all on one plate, and Jordan, who conducted these experiments, concluded that their survival was accidental, since they were not found during the preceding six days.

In the Illinois River, fourteen parchment sacs containing the raw river water and typhoid bacilli were suspended in, and thus exposed to the influence of, the river water. The number of organisms in the river water varied from 60 to 500, while the number of typhoid bacilli added ranged from 500 to 20,000 per c.c. In only one case were any typhoid bacilli recovered after the third day. In this case, a single colony giving all the cultural and other tests of the typhoid bacillus was isolated ten days after inoculation.

These three investigations, independently conducted, but all using the same strains of typhoid bacilli, yielded strikingly uniform results. The authors conclude that 'under conditions that probably closely simulate those in nature, the vast majority of typhoid bacilli introduced into the several waters studied, perished within three to four days,' and that 'it is theoretically possible that specially resistant cells may occur which are able to withstand for a longer period the hostile influences evidently present in water. Our experiments, however, show that if such resistant individuals exist they must be very few in number, and constitute only a small fraction of the bacilli originally entering the water.'

Still more recently, Russell and Fuller (Russell and Fuller, 1906) have reinvestigated this question in view of

the striking differences between these results and those of earlier investigators. Parchment, celloidin, and agar sacs were used, and one of the typhoid strains employed in the previous investigation was again used.

In four series of examinations in which *B. typhosus* was exposed to the action of flowing lake water (a spring-fed inland lake) the longevity of the organism varied from eight to ten days, 'agreeing quite closely with the experiments previously reported on Lake Michigan water under similar experimental conditions.' Where the typhoid bacillus was exposed directly to the action of sewage bacteria, its longevity was greatly diminished, three to five days being the longest time during which the organism could be recovered. When the typhoid organism was exposed to the diffusible products of sewage bacteria, and not to the direct action of the organisms themselves, the longevity of the bacillus was increased. This was carried out by filling the typhoid-infected sacs with lake water instead of sewage, and then suspending them in flowing sewage.

When typhoid-infected sacs filled with sewage were exposed to the action of water, the longevity of the typhoid bacillus was the same (five days) as when the liquid surrounding the sac was sewage. This suggests that the question of longevity is dependent more upon actual contact with sewage bacilli, than upon contact with by-products capable of diffusion through these permeable membranes.

Among other conclusions, they remark that 'the uniformity noted in the results obtained in this investigation, and their confirmation of the work of the preceding year on the waters of Lake Michigan and the Chicago Drainage Canal, would now seem to warrant the definite conclusion that the longevity of the typhoid bacillus in waters is materially affected by the germ content of its surroundings. In waters highly polluted with saprophytic bacteria, such as is the case in sewage, this disease organism is unable to survive for more than a few days (three to five days in the experi-

ments described), a period of time materially shorter than that which is noted in normally unpolluted waters.'

These experiments show a much more limited viability for the typhoid bacillus in water than seemed probable from the earlier work on this subject, while they are not altogether in accord with epidemiological experience. The extreme thoroughness with which they were evidently conducted entitles them to very careful consideration. They are of particular interest and importance from the standpoint of sewage treatment and the likelihood of typhoid bacilli surviving bacterial and other methods of sewage purification.

The factors influencing the survival of typhoid bacilli in water are probably complex, and not simply a matter of food-supply, temperature, and influence of other organisms. Recently Whipple and Mayer (Whipple and Mayer, 1906) have brought forward evidence that the presence of dissolved oxygen is important, and enables the typhoid bacillus to maintain its vitality longer than is possible when oxygen is absent.

In particular, the conditions under which the typhoid bacilli gain access to water are probably of the utmost importance. It is well known, for example, that naked typhoid bacilli—*i.e.*, in laboratory cultures—are much more readily killed by disinfectant solutions than those in fæces. This may be only a question of physical protection, but it is certainly unjustifiable from experiments with the typhoid bacillus in cultures to deduce conclusions as to the same bacillus in fæces. It by no means follows that because typhoid bacilli from cultures in the above experiments lived only for a very few days, typhoid bacilli in fæces would all have been killed in the same period. It may be a true statement, but the experiments with naked germs do not warrant our drawing the conclusion as an undoubted one, or even as a probability.

The experiments made upon the vitality of the typhoid

bacillus in water, soil, and sewage show clearly that under saprophytic conditions this organism is at a disadvantage, and cannot compete successfully with the other bacteria present, which are in what is to them a more natural environment.

Frost (Frost, 1904) has recently made a careful study of the antagonism of certain saprophytic bacteria for the typhoid bacillus. He investigated this antagonism in a number of ways, but most frequently by the use of collodion sacs attached to test-tubes open at both ends. The interior of the sac was filled with a suitable medium, and then inoculated with the typhoid bacillus. It was then suspended in water, or broth, inoculated with the materials under investigation. He showed that soil bacteria have a markedly inhibitory action upon the growth of the typhoid bacillus, and this was present whether the material added to the outer fluid was pure or polluted soil. In some cases this antagonism was not marked for several weeks, but usually within one week a reduction of 95 to 100 per cent. of the typhoid bacilli took place, while in the control they remained very numerous. In regard to water bacteria, an antagonism was exhibited when these bacilli were allowed to multiply in the outer water, but when the typhoid bacilli were exposed in the sacs to the running water of the lake no influence due to water bacteria was exerted. Throughout all the experiments the strains of *B. typhosus* used were not recently isolated. This fact detracts somewhat from the value of the results obtained. Frost concluded that there is no evidence to show that the antagonistic substances exist ready formed in the soil or water, but rather that the antagonism depends upon the rapid development of the germs in the immediate presence of the typhoid bacillus. He isolated a number of species of bacteria which were definitely antagonistic to the growth of *B. typhosus*—*e.g.*, the potato bacillus, *Proteus vulgaris*, *B. fluorescens liquefaciens*, and *B. fluorescens putidus*. Frost found that the energy with which the antagonistic substances act depends on the temperature.

At 38° C. the action is very pronounced. At the temperature of the ice-chest (10° to 12° C.), the typhoid germ may grow in the by-products of the other germs, which at higher temperatures are quickly fatal to it.

The antagonistic action was found to be due not to the peculiarity of any single typhoid strain, but was equally noticeable for all the three strains examined.

Other Members of the Eberth Group.

Knowledge with regard to other organisms of the Eberth group is at present very limited. It is probable that they form an extended series. Apart from the organisms which simulate the typhoid bacillus in their cultural characters, and which have been isolated from soil and water, only two definite named organisms are known and require mention—*B. dysenteriæ* and *B. fæcalis alcaligenes*.

B. Dysenteriæ.

First isolated by Shiga in 1897 during an epidemic of dysentery in Japan. Kruse, in 1899, found an apparently identical bacillus in an epidemic of dysentery in Westphalia; and Flexner isolated a very similar bacillus from dysentery cases in the Philippines. The organism, or organisms—for undoubtedly there are more than one—has since been found by a number of observers. There are two main types—the Shiga type, not fermenting mannite, and the Flexner-Harris type, which does ferment mannite —but others have been described.

The characters of this group of bacilli, as given below, are largely taken from a recent valuable paper by Firth (Firth, 1904). They are all cylindrical bacilli with rounded ends, about 1 to 3 μ long, and without spores. Decolourized by Gram's method. There is not complete agreement as to motility, but apparently it is exhibited under certain conditions, and flagella can be demonstrated.

On gelatine media they grow very similarly to the

typhoid bacillus. They ferment glucose, with the production of acid, but not gas; lactose and saccharose are unaffected. Some races produce indol, others do not. In litmus-milk there is never coagulation, but there is a production of alkali, always slight in amount, with or without preliminary acid formation.

As regards maltose, mannite, and galactose, their action is variable, one group fermenting them, while the other main group does not.

The different dysentery bacilli, and particularly the mannite-splitting group, closely resemble therefore the typhoid bacillus, the essential difference being their failure to be agglutinated by a typhoid serum, while they are acted upon by dysentery serum.

As far as the writer is aware, undoubted dysentery bacilli have never been found in polluted water, but this brief description is included because they have to be distinguished from the typhoid bacillus, while as intestinal organisms they must have frequently gained access to water-supplies, and may in the future be isolated from them.

B. Fæcalis Alcaligenes (= *B. Alcaligenes*).

Isolated by Petruschky (Petruschky, 1896) from typhoid stools. It closely resembles the typhoid bacillus in many of its cultural characters, but according to Petruschky it is readily distinguished from it by its growth in milk, by its failure to give Pfeiffer's immunizing reaction, and by giving, after a few days, a brown growth on potato.

Ford (Ford, 1903) found this organism in fourteen out of fifty examinations of the alimentary tract of cadavers. It was found in the rectum alone in five cases, in the cæcum alone in two cases, in the duodenum alone in three cases. He also found it in both rectum and stomach, in rectum and duodenum, in cæcum and duodenum, and in duodenum and stomach in other cases.

Ford describes its characters as follows (abbreviated): Bacilli resembling the typhoid bacillus in morphology. Actively motile; no spores.

Agar Slant.—White glistening growth limited to line of inoculation, not spreading.

Broth.—Heavy thick scum on the surface, the broth itself being fairly clear and often free from sediment.

Gelatine Stab.—Abundant growth along line of inoculation; no liquefaction.

Gelatine Surface Colonies.—Round, translucent, with nail-form appearance.

Potato.—Growth varies from a scanty white to an abundant dirty yellowish or brownish mass covering the entire surface of the potato. Growth rarely reddish-brown.

Dextrose, *saccharose*, and *lactose* not fermented.

Nitrates reduced to nitrites.

Litmus-Milk.—No stage of preliminary acidity, but rapid alkali production; within forty-eight hours the milk turns blue. No coagulation.

Indol.—Produced rarely in old cultures.

Pathogenicity.—Non-pathogenic to mice, guinea-pigs, or rabbits.

To this description may be added that it is not stained by Gram's method, the colonies on Drigalski-Conradi media plates are blue, and it is not agglutinated by dilute antityphoid serum. The thick scum in broth described by Ford is by no means constant.

There is no doubt a group of organisms which can be classed under this head rather than one definite organism.

Klein (Klein, 1905) records that he has found it in a typhoid stool, and in two, out of five, samples of sewage from St. Bartholomew's Hospital.

The writer has isolated this organism from the stools of a typhoid patient, in which it was extremely abundant, being certainly more numerous than 1,000,000 per gramme; from a fly; from river mud near a sewage outfall; and recently

from two shallow wells, both of which were on bacteriological grounds satisfactory, neither showing the presence of *B. coli* or streptococci in 50 c.c. of the water. The strains from the mud and excreta were both non-pathogenic, the other three were not tested. These five organisms were tested as regards their agglutination properties with a powerful antityphoid serum. The organism from mud gave a marked positive reaction in 1 per cent. dilution (positive in fifteen minutes), and a nearly complete reaction with a 0·1 per cent. dilution (one hour); but on retesting a month later, no reaction took place with either dilution. The others failed to react both with this and with a paratyphoid serum. All of them failed to ferment dulcite, mannite, or saccharose, all gave a negative neutral red reaction, all but one produced indol in peptone solution.

They all agreed with Ford's description in that in no case was there any preliminary acid production in litmus-milk, but they differed in that the alkalinity was only slowly produced, not being obvious under three to four days' incubation, although very marked after fourteen days.

Terburgh (Terburgh, 1906) investigated the contaminated canal water of Amsterdam, and isolated fourteen organisms which belonged to the group of *B. fæcalis alcaligenes*. Of these, only three produced a distinct surface scum in broth. They all failed to agglutinate powerful antityphoid serum, and Terburgh concluded that it is quite possible to distinguish them from the typhoid bacillus.

Porcille (Porcille, 1905) immunized various animals with eight different typhoid races. *B. fæcalis alcaligenes* was not agglutinated by any of the eight typhoid sera, in dilutions of 1 to 20 or greater. On the other hand, the serum of an animal immunized against a *B. fæcalis alcaligenes*, which with that organism gave a positive agglutination reaction in dilutions of 1 to 2,000, also agglutinated all the five typhoid bacillus strains tested in dilutions of 1 to 20, four in dilutions of 1 to 50, and one partially in a dilution of 1 in 100.

Recently Altschüler (Altschüler, 1904), and subsequently Doebert (Doebert, 1905), have claimed that they have converted a member of this group, by passage through three guinea-pigs, into a bacillus indistinguishable from a genuine typhoid bacillus, and agglutinated by antityphoid serum in a dilution of 1 to 15,000. Terburgh (*op. cit.*) was unable to confirm this. Obviously such a statement requires ample confirmation before it can be accepted.

This group is easily distinguished from the Gärtner group, with which Durham at one time placed it, by, amongst other characters, its failure to ferment glucose, absence of neutral red reaction, and absence of preliminary acid production in milk.

The fact that undoubted *B. typhosus* strains may sometimes produce alkalinity in milk brings the two organisms nearer as regards their cultural characters, but the typhoid bacillus always produces initial acidity in milk, and produces acid in glucose and in mannite media, which *B. fæcalis alcaligenes* does not do.

CHAPTER VIII

Other Intestinal Bacteria

The Streptococcus Group.

The name streptococcus being only a description of a morphological type, must obviously include a number of different organisms. Many different kinds of streptococci have been described, and much discussion has taken place as to how far the described varieties are separate species or mere variants of a common type.

From the point of view of the water bacteriologist, it is of importance to consider whether streptococci as a class have to be considered as indicators of pollution, or only certain special kinds of streptococci.

The methods in use to classify streptococci cannot be considered very satisfactory. Their morphological characters, staining properties, action upon gelatine and litmus-milk, characters of the growth upon agar and gelatine media, agglutination properties and pathogenicity, yield valuable differentiating results, but do not sharply mark off different species of streptococci, or serve to differentiate races which obviously play different parts in disease causation.

Gordon (Gordon, 1903-1904) has recently introduced a fresh series of tests for the differentiation of streptococci, depending mainly on the power of the races isolated to ferment various sugars and alcohols. The nine tests selected by him for routine employment were: (1) The question as to the clotting of litmus-milk in three days at 37° C.

(2) The reduction of neutral red broth during anaërobic incubation for two days at 37° C. (3) The production of an acid reaction in three days at 37° C. when cultivated, under aërobic conditions, in slightly alkaline broth containing 1 per cent. of saccharose. (4) Ditto, but lactose. (5) Ditto, but raffinose. (6) Ditto, but inulin. (7) Ditto, but salicin. (8) Ditto, but coniferin. (9) Ditto, but mannite.

These differentiating tests promise results of great practical utility, but they have not been, as yet, sufficiently investigated to form a firm basis for classification.

The proper way to utilize the streptococcus group, as a means of indicating sewage and excretal pollution, is obviously to ascertain the varieties of streptococci present in such substances, and to consider how far these varieties fulfil the conditions of satisfactory bacterial indicators of contamination. Horrocks and Houston have both endeavoured to do this.

Horrocks (Horrocks, 1901, *a*, p. 115 *et seq.*) described five varieties of streptococci from sewage. These five organisms showed almost every variation, except that they were all stained by Gram's method and were all non-pathogenic to guinea-pigs. Three formed short chains, one chains of medium length, and one long chains; four did not liquefy gelatine, one slowly liquefied it; four coagulated milk, one did not; two produced indol, three did not; three grown in litmus-whey at 37° C. and tested at the end of a week showed 13 to 14 per cent. of $\frac{N}{10}$ alkali, one only 2·4 per cent., while the fifth produced no acid. No figures as to the relative frequency of the different varieties were given, and no one common type was found.

Houston has isolated a large number of streptococci from sewage and from polluted waters, and found great variation in their characters.

From London sewage (Houston, 1900) he isolated many varieties, finding considerable differences. He described, however, one kind (not an individual species) as being the

type of streptococcus most commonly present in crude sewage and in the effluents from the coke-beds. This type he called the *sewage streptococcus*, and found it in numbers usually more than 1,000 per c.c. in both crude sewage and sewage effluents.

It has the following characters:

Grows well at blood heat; stains by Gram's method; chains of cocci usually short.

Agar and Gelatine Plate Cultures (under a low power).—The colonies are small and transparent. They are nearly circular in shape, with a *clean* edge. The granulation is faint. The colonies in agar are usually more granular and darker looking than in gelatine cultures. The gelatine is not liquefied.

Streak Cultures (agar at 37° C. and gelatine at 20° C.).—The growth usually shows itself as a delicate white film, which on close observation is seen to be made up of numerous separate, minute, transparent-looking colonies. The gelatine is not liquefied.

Broth Cultures (37° C.).—Abundant diffuse cloudiness. On gently shaking the tube a viscous white deposit rises from the foot of the tube in a spiral form.

Litmus-Milk Cultures (37° C.).—Acidity, but usually no clot. Probably non-pathogenic to mice.

In an investigation upon human excreta, Houston (Houston, 1902-1903, *a*) isolated seventy different streptococci from seventeen samples of fæces. Of these, 91 per cent. formed short to medium chains in broth cultures, the majority forming short chains; 85 per cent. produced uniform turbidity in broth cultures; 81 per cent. caused acid clotting in milk cultures (from later work Houston concludes this percentage is too high); 86 per cent. were non-pathogenic to mice, if the doubtfully pathogenic be included with the pathogenic. Considering cases where only one of the two mice died as non-pathogenic, and counting as pathogenic only those in which both mice

succumbed, 96 per cent. of the streptococci were non-pathogenic.

Houston, grouping these results, finds that the type of streptococcus most commonly met with in human excreta, and which he calls *fæces streptococcus*, has the following characters :

Short to medium, but on the whole short, chains.

Uniform turbidity in broth.

Acid, and solid clot within five days at 37° C. in litmus-milk cultures.

Non-pathogenic to mice.

In a subsequent report (Houston, 1903-1904) examined nineteen additional normal human stools, and isolated and studied over 300 streptococci. Streptococci were present in numbers from 100,000 to 1,000,000 per gramme of fæces. Seventy-five per cent. produced diffuse cloudiness in broth cultures. All those examined were non-pathogenic. None of the streptococci tested reduced nitrates to nitrites, and none of them liquefied gelatine.

The greater part of the work upon these streptococci consisted in testing their action upon certain sugars and alcohols for acid production, as recommended by Gordon. The streptococci were examined in three series of 100 each. The results of these differentiating tests is shown in the following table :

Test.	Series I.	Series II.	Series III.	Average of Results per Cent.
	Per Cent. Positive.			
Salicin	96	91	91	92·67
Saccharose	89	84	86	86·34
Lactose...	66	78	85	76·34
Litmus-milk (acid and clot) ...	51	55	79	61·67
Neutral red broth	35	35	48	39·34
Raffinose	62	8	26	32·00
Mannite	7	27	39	24·34
Inulin	12	0	2	4·67

The change in these substances consists in those which yield a positive result, in the production of acid only, no gas being produced with any of them.

These 300 streptococci fall into no less than forty separate groups on the basis of the above tests. Houston, in this connection, remarks (p. 512): 'It is obvious that the streptococci in fæces constitute a race of microbes differing widely in their biological attributes. Unfortunately, with so many groups to select from, a particular streptococcus or pseudo-streptococcus derived from a non-fæcal source could hardly fail to fall under one or another of the forty groups of fæcal streptococci.'

Clearly, then, these sugar-alcohol tests, while full of promise, do not offer any means by which streptococci from fæces can be distinguished with any certainty from those derived from other sources. It should be noted that the different sugar-alcohol tests are much more definite and reliable when applied to *B. coli*. With that organism reliance is placed primarily on gas production and only quite secondarily on acid formation. Streptococci produce either no gas, or no visible amount of it, in any medium.

The characters of the sewage and the fæcal streptococcus are shared by many streptococci derived from quite different sources.

Bacillus Enteritidis Sporogenes.

This is an anaërobic spore-bearing bacillus isolated by Klein in 1895 from the evacuations of patients suffering from an epidemic of diarrhœa in St. Bartholomew's Hospital. It was also isolated from the milk consumed by these patients.

From the point of view of the bacteriological examination of water, this organism owes its importance to the fact that it is prevalent in sewage and in excreta, and has on this account been used as a bacterial indicator to show the pollution of water-supplies by these substances.

Its essential characters, as described by Klein (Klein, 1901-1902), are given in the following table:

I. *B. butyricus.*	II. *B enteritidis sporogenes.*	III. *B. cadaveris sporogenes.*
1. Cylindrical rods, on the average 2·5 to 3·5 μ long, 0·8 to 1·25 μ broad; stains well by Gram's method; some individuals motile.	1. Same as in I.	1. Cylindrical and thread-like; thinner and longer than I. and II.; very motile; stains by Gram's method.
2. Spores oval; stain after the several methods; situated in the middle of the rods more or less.	2. Same as in I.	2. Spores oval, terminal, drumsticks; stain after usual methods.
3. Grows well on the surface of ordinary gelatine as a translucent mass of convoluted threads; does not liquefy the gelatine.	3. Softens rapidly the gelatine; slowly liquefying.	3. Rapidly liquefying; putrid odour; numerous spores formed.
4. In stab in gelatine, forms spherical colonies with numerous horizontal filamentous projections; not liquefying; forms much gas.	4. Much gas; spherical colonies; without filamentous projections; slowly liquefying.	4. Much gas; rapidly liquefying; putrid odour.
5. On the surface of agar, gray, round, flat colonies; margin thin and much crenate; no spores.	5. Same as in I.; few crenations; no spores.	5. On the surface of agar forms thready, branched colonies; with or without finely granular plate; rapidly forming spores.
6. In stab in agar, forms characteristic bundles of threads projecting laterally from the growth in the stab; much gas; no spores.	6. Little tendency for forming lateral branchings; much gas; no spores.	6. Much gas; rapidly forming spores; conspicuous masses of threads growing out of stab.
7. In milk, rapid separation of acid whey and flocculi of casein; smell of butyric acid; no spores; much gas.	7. Same as in I.	7. Milk is slowly decomposed; putrid odour; much gas; rapidly forming spores.
8. Grows well on serum; very slow softening.	8. Grows well; slowly liquefying; some spores formed.	8. Rapidly liquefying; putrid odour; rapidly forming spores.
9. Not pathogenic for rodents.	9. Virulent for rodents.	9. Not pathogenic for rodents.

The virulence of this organism is subject to considerable variation, to some extent dependent upon its source. Thus Klein records the extremely high virulence of the spores of this bacillus when derived from Hendon sewage as contrasted with the lesser virulence of similar spores from cases of diarrhœa and from typhoid stools.

Typical virulence is exhibited if 1 c.c. of the clear, or slightly turbid, whey of a typical milk culture, one to two days' old, when injected under the skin of the groin of a guinea-pig of 200 to 300 grammes body-weight, causes the following changes found after death, which takes place usually within twenty-four hours: The hair readily strips off on the inoculated side near the site of inoculation, the skin beneath being green and gangrenous. The subcutaneous tissue is largely destroyed, and its place filled by an offensive sanguinous exudation, containing numerous bacilli with rounded ends, staining by Gram's method. No spores are found, but if the examination is not made until the day after death, numerous spores, free and in the bacilli, are found.

The changes in milk grown anaërobically are characteristic, and since this is the test which is invariably used for the diagnosis of this bacillus, a detailed description is given. The changes usually take place within twenty-four hours, or two days at the latest. Milk recently boiled to expel the air should be used, and incubated under anaërobic conditions. Gas is abundantly formed, and the cream is torn or altogether dissociated by the bubbles, so that the surface of the medium is covered by stringy, pinkish-white masses of coagulated casein, enclosing gas bubbles. Beneath is a colourless, thin, clear or slightly turbid whey, with some casein entangled here and there and at the bottom of the tube. The whey has a markedly acid reaction and a pronounced butyric acid odour, and contains numerous bacilli which, according to Klein's later description, never contain spores.

This bacillus resembles more or less closely several other anaërobic organisms. The essential differences between two of these, *B. butyricus* and *B. cadaveris sporogenes*, are given in the table on p. 115.

B. butyricus closely resembles *B. enteritidis sporogenes* in its morphological and cultural characters, and Klein at first stated that from these alone no diagnosis could be made between them, and that the only difference was that *B. butyricus* was non-pathogenic to rodents, while *B. enteritidis sporogenes* was highly virulent. Later careful work by the same investigator (Klein, 1901-1902) showed that they could be distinguished culturally by their action on gelatine. When grown on sloped gelatine, incubated anaërobically at 20° to 21° C., and examined after eight days, *B. butyricus* shows a gray translucent growth, with no trace of liquefaction, while *B. enteritidis sporogenes* grows as whitish-gray masses and granules, softening and liquefying the gelatine. Examined later, these changes are still more apparent, while *B. butyricus* remains unaltered, and does not cause any trace of liquefaction, even after weeks and months. Klein states that the difference is still more strikingly shown in stab culture in urine-gelatine.

B. cadaveris sporogenes is another anaërobic organism from the intestine, but one that is readily distinguishable from *B. enteritidis sporogenes*. Its characters are described in the table on p. 115. The terminal spore, the cultural differences in milk and gelatine, and the non-pathogenicity, are distinctive differences.

Another organism which has to be considered in this connection is *B. aerogenes capsulatus* of Welch. According to Hewlett (Hewlett 1902, p. 331) this organism is probably identical with Klein's *B. enteritidis sporogenes*. Hewlett states that the only difference between them is that, according to Klein, *B. enteritidis sporogenes* is motile and flagellated, while *B. aerogenes capsulatus*, according to Welch, is non-motile and non-flagellated.

The diagnosis of *B. enteritidis sporogenes* is invariably made, in the first place, by the characteristic change in milk. Confirmation is sought by the guinea-pig inoculation test. The changes produced in milk by *B. butyricus* are indistinguishable from those produced by *B. enteritidis sporogenes*. It follows, therefore, that the milk test by itself is not a measure of the number of spores of *B. enteritidis sporogenes* alone, but of the number of spores of that organism together with the spores of *B. butyricus*, and those of any other undiscovered organisms which may be present, which produce a similar change in milk. A measure of the numerical distribution of *B. enteritidis sporogenes* alone can only be obtained after inoculation experiments have been made, unless the more recent cultural distinctions described by Klein are made use of, and for this purpose the bacillus must be isolated in pure culture—an impossible task in routine work.

For routine work, a test involving systematic animal inoculations is inconvenient and frequently impracticable, and as a matter of fact the value of this organism as a test for excretal contamination depends mainly upon the value of the milk reaction alone.

Looked at from this point of view, the distribution of *B. butyricus* is of great importance. Klein (*ibid.* p. 412) finds that sewage and many other filth materials contain the spores of both microbes, and that 'sewage contains the spores of *B. butyricus* as a rule more abundantly than those of *B. enteritidis*.'

In regard to *B. cadaveris sporogenes*, Klein (*ibid.*, p. 415) remarks: 'The spores of this microbe are present in the contents of the alimentary canal of man and other animals; they are present in fæcal matter, in manure, street dust, sewage, and all similar filth—that is to say, the spores of this anaërobe occur almost in the same materials in which the spores of *B. butyricus* and *B. enteritidis sporogenes* occur.'

It is important to recognise these facts, and to realize that

the characteristic milk change is an indication of the presence of certain spores, presumably of one or other of these organisms; it is not a measure of *B. enteritidis sporogenes* alone. It is, perhaps, in this sense comparable rather to the presumptive tests for *B. coli* than to tests including the actual isolation of the organism.

Spirillum Choleræ Asiaticæ.

Koch's comma bacillus. Isolated first by Koch from the stools of cholera patients, and now generally accepted as the cause of cholera. Its essential characters are the following:

A curved organism about 1·5 to 2·0 μ long, which occurs usually singly, but sometimes in pairs curved in opposite directions, forming an **S**-shaped figure. In liquid media it may grow into long spiral filaments. Involution forms are frequently met with in cultures, particularly old cultures. When this occurs the size and shapes met with are very varied, including clubbed forms, short, thick organisms looking like cocci, twisted filaments, etc. No spores are produced. The cholera vibrio is very actively motile. It has one flagellum, and this is terminal.

It stains readily by the ordinary stains, but does not retain the stain in Gram's method.

It grows readily on all the ordinary media, and best at 37° C.

It is strictly aërobic.

Broth.—Uniform turbidity; very rapid growth, with production of thin pellicle on the surface.

Gelatine Stab.—Growth all along the line of inoculation, but most marked near the surface. The gelatine begins to show liquefaction as early as forty-eight hours. This rapidly proceeds, the liquefied gelatine producing a funnel-like appearance, which gradually extends downwards until the whole tube is involved. The rate of liquefaction is slower than that of several closely allied spirilla.

Gelatine Plate Colonies.—Just visible after twenty-four hours as small white points. After forty-eight hours they are considerably larger, and under a low magnification appear as rounded colonies with an irregular undulating margin. The surface is coarsely granular, giving the so-called 'ground-glass' appearance. As growth progresses liquefaction occurs, the colonies sink into small cups of liquefied gelatine, and are surrounded by an ill-defined halo, the whole appearance being fairly characteristic.

Potato.—If slightly acid, no growth takes place at 22° C., but if alkaline a yellow-brown growth results.

Cholera-Red Reaction.—This is an indol reaction, but differs in that this organism produces a nitrite as well as indol, so that it is only necessary to add the acid. Both nitrite and indol are rapidly produced. To perform this test a few drops of pure—*i.e.*, nitrite-free—sulphuric acid are added to a twenty-four hours' peptone-water culture of the cholera vibrio. The characteristic red colour develops.

This reaction, although characteristic of this organism, is not peculiar to it, as it is also given by some other spirilla.

Litmus Milk.—Active growth takes place with production of acid and coagulation of the casein.

The agar plate colonies are not characteristic.

Pathogenicity.—Intraperitoneal injection of broth cultures into guinea-pigs and mice usually causes death with peritonitis.

In addition to the cultural characters, Pfeiffer's test and the agglutination reactions are valuable means of identification.

Pfeiffer's Test.—The serum of an animal immunized against the cholera vibrio, to as high a grade as possible, is used to provide an anticholera serum.

A loopful (2 milligrammes) of an eighteen hours' old agar culture of the suspected vibrio, incubated at 37° C., is added to 1 c.c. of ordinary broth containing 0·01 c.c. of the

anticholera serum. The mixture is injected into the peritoneal cavity of a guinea-pig weighing 200 grammes. By means of a glass pipette a little peritoneal exudation is removed at the end of twenty minutes, and again after one hour. This is examined in hanging-drop preparation. If the organism is the true cholera spirillum, a positive reaction is obtained. This is shown by the vibrios having become broken down and transformed into granules.

This lysogenic action is not met with if the vibrio is not the true cholera organism, the vibrios remaining alive and actively motile.

A control must at the same time be performed by the injection into the peritoneal cavity of another guinea-pig of a loopful of the culture, mixed with 1 c.c. of broth containing 0·01 c.c. of *normal* serum, from the same species of animal as that from which the anticholera serum was obtained. The vibrios under these circumstances should remain motile and unaffected when withdrawn and examined in hanging drop after twenty minutes and one hour.

Agglutination Tests.—Cholera vibrios are agglutinated by the serum of an animal immunized by repeated injections of a virulent cholera culture. Precautions as to dilution, etc., have to be observed just as in the corresponding typhoid agglutination tests.

According to Kolle and Gotschlich (Kolle and Gotschlich, 1903), cultures not more than eighteen hours old should be employed, and it is absolutely necessary to make the following controls :

1. With the suspected culture, and with *normal* serum of an animal of the same species as that used to obtain the anti-cholera serum, but in tenfold stronger concentration.

2. With the suspected culture and the diluting fluid alone.

3. With a known cholera culture, of the same age as the culture under investigation, and with the anticholera serum.

A high-grade serum must be employed, and a series of different dilutions should be used—*e.g.*, from 1 : 100 to 1 : 2,000.

Kolle and Gotschlich (*ibid.*) in 1903 investigated a large number of cholera-like vibrios obtained in Alexandria from the excreta of patients suffering from cholera, or suspected of having that disease. The tests upon which most reliance could be placed for the diagnosis of true cholera organisms were carefully investigated and studied. Some of the allied vibrios could not be distinguished by any morphological or cultural characters from the cholera vibrio, while only in certain cases was the pathogenicity test of use as a means of differentiation.

On the other hand, some of the vibrios possessed two, four, six, or more flagella, while the genuine cholera organism has always only one.

They found that a rapid and certain differential diagnosis between these organisms could be made by means of agglutination reactions and the bacteriolytic (Pfeiffer's) test.

The allied vibrios were not agglutinated by a high-grade cholera serum, and no bacteriolytic action was exerted when they were introduced, mixed with cholera serum, into the peritoneal cavity of animals. Each of the twenty-one cholera-like vibrios was used to inoculate a rabbit. The serum of these animals showed neither agglutination action nor bacteriolytic properties towards genuine cholera cultures, but only against the vibrios with which they had been inoculated and a few others with which they were identical.

Allied Vibrios.

A considerable number of different vibrios have been isolated from various sources, including human excreta and water-supplies, and a good deal of attention has been paid to their diagnosis from the cholera spirillum.

Now that Pfeiffer's test and the agglutination reactions are available, the differentiation of these allied organisms is

comparatively easy, but so closely do some of them resemble in morphology and cultural characters the vibrio of cholera, that, without these tests, it is frequently very difficult, and sometimes impossible, to discriminate between them.

The following include the more important of such vibrios:

Vibrio Metchnikovi.—Isolated by Gamaleia at Odessa, where it was found post-mortem in a number of fowls which had died of an epidemic disease associated with gastro-enteritis. It was subsequently found by Kutscher in river water.

Morphologically it closely resembles the cholera spirillum, but is usually somewhat thicker and more distinctly curved. It is actively motile, and possesses only one flagellum.

In gelatine stab and on gelatine plates its growth is very similar to that of the cholera spirillum under corresponding conditions, except that it grows rather faster and causes more rapid liquefaction.

It gives the cholera-red reaction.

Its other cultural characters are very similar to those of the organism of cholera.

Its pathogenic properties, however, are dissimilar, and afford a certain means of diagnosis.

A quite small quantity, such as would remain on the point of a needle, introduced into the pectoral muscles of a pigeon sets up a quickly fatal septicæmia, causing the death of the animal in from eight to twenty-four hours. The vibrios are present in enormous numbers at the site of inoculation. The inoculation of pigeons with approximately the same number of cholera vibrios produces practically no result.

Guinea-pigs inoculated subcutaneously usually die within twenty-four hours of acute general septicæmia.

Metchnikoff's vibrio does not give Pfeiffer's test when a high-grade *cholera* serum is used, but the serum of animals which have been treated with this vibrio possesses bactericidal properties for that organism.

Finkler and Prior's Spirillum.—Found by these observers at Bonn during an epidemic of cholera nostras. It possesses to-day only an historic interest.

Morphologically it is very similar to the cholera spirillum, but culturally it shows marked differences. In gelatine stab it grows much more rapidly and with more rapid liquefaction, liquefaction being visible generally within twenty-four hours. The gelatine plate colonies show a proportionately quick growth.

The cholera-red reaction is not given.

The virulence of this organism is much lower than that of the cholera vibrio.

Deneke's Spirillum.—Isolated by Deneke in 1885 from old cheese.

Morphologically it closely resembles the cholera vibrio, but culturally it is easily distinguished by the appearance of its gelatine plate colonies, which are yellow and liquefy more rapidly, while the cholera-red reaction is not given. Feebly or not at all pathogenic.

Vibrio Phosphorescens.—This name really includes a group of vibrios isolated in 1893 by Dunbar and Rumpel from the water of the Elbe, Rhine, Havel, and Spree. At first they were supposed to be cholera vibrios, but Kutscher showed that cultures exhibited phosphorescence, especially at a temperature of 22° C. Many were pathogenic to animals. Cholera-red reaction given. They did not give Pfeiffer's test.

Spirillum Massowah.—Isolated by Pasquale from a small epidemic of cholera at Massowah. For some time it was supposed to be a genuine cholera vibrio. It differs, however, in that it possesses four flagella, it liquefies gelatine more slowly, while its pathogenic properties are very similar to those of *Vibrio Metchnikovi.* It also shows no specific immunity reaction with cholera serum.

The *Vibrio Ghinda, Vibrio Danubicus, Vibrio aquatilis, Vibrio Berolinensis,* were all isolated from water-supplies. They

can be distinguished from the cholera vibrio by minor cultural characters, and particularly by their failure to give Pfeiffer's test.

In addition to these named organisms, a large number of vibrios have been isolated from river water. Sanarelli obtained from the water of the Seine and Marne thirty-two vibrios; Wernecke isolated vibrios from the Elbe, and Dunbar vibrios from the rivers Spree, Elbe, Havel, and Oder. Numerous other workers have also found vibrios in river water.

Some of these organisms resembled the cholera spirillum closely in their morphological and cultural characters, and a number were pathogenic to animals.

Considering the matter broadly, they may be classed into two groups:

1. Vibrios which can be fairly readily differentiated from the cholera spirillum by morphological and cultural characters alone.

Most of the vibrios isolated from water belong to this class. The gelatine colonies and growth in gelatine slab are sufficient to distinguish from the cholera vibrio in many cases. Some possess two or more flagella, others fail to give the cholera-red reaction, etc., etc.

For example, of the thirty-two vibrios isolated by Sanarelli, only four gave the nitroso-indol (cholera-red) reaction.

2. Vibrios which show no definite and diagnostic differences, as regards morphological and cultural characters, from the true cholera organism.

The diagnosis of this class of organisms is much more difficult, and it is necessary to use both the agglutination reaction and Pfeiffer's test for this purpose.

The existence in water-supplies of vibrios resembling the cholera spirillum shows how essential it is (as in the case of the typhoid bacillus) to use *all* the available diagnostic tests before any organism isolated from a water-supply can be said to be the true vibrio of cholera.

CHAPTER IX

The Content of Various Waters with regard to the Presence of B. Coli, B. Enteritidis Sporogenes, and Streptococci

In Chapter II. an account was given of the number of bacteria in normal waters which will develop upon ordinary nutrient media. In the present chapter examples are given of results obtained, showing the frequency of the occurrence of *B. coli*, *B. enteritidis sporogenes*, and streptococci, in different kinds of waters.

The investigations referred to are mainly English, as likely to be of more value to workers in this country.

H. Chick (Chick, 1900), investigating the distribution of *B. coli*, found this organism absent in 0·5 and 1·0 c.c. in eleven samples of rain water. In moorland streams (1 c.c. being examined in each case), in one series of 38 samples, *B. coli* was found in 7, all the 7 being from streams which showed probable sewage contamination on topographical investigation; and in a second series from another watershed, of 53 samples, this organism was found in 11 cases (in 1 c.c.), while in 4 its presence was doubtful.

Houston carried out two series of investigations upon the bacterial content of the shallow-well waters of Chichester. For the first report (Houston, 1900-1901, *b*) 30 samples were examined from 14 different sources, 10 being from reputed typhoid fever areas and 4 from non-fever areas. All the samples, except one, were from covered wells provided

with pumps. For the second report (Houston, 1901-1902, *b*) 20 samples were examined, 10 being from reputed typhoid fever areas and 10 from non-fever areas. No topographical details were supplied, and no essential bacteriological differences were made out between the waters in the fever and non-fever areas. The term *B coli* was used in a very broad sense in these investigations, and included allied forms.

The following results were obtained for the two investigations:

Number of samples examined	= 50
Coli-like bacilli present in 0·1 c.c.	in 10
,, ,, ,, 10 c.c.	in 28
,, ,, ,, 100 c.c. or less	in 40
No coli-like bacilli in 100 c.c.	in 10
Streptococci present in 10 c.c.	in 6
,, absent in 10 c.c.	in 43
B. enteritidis sporogenes present in 100 c.c.	in 4
,, ,, ,, 200 c.c.	in 7
,, ,, absent in 200 c.c.	in 43

The presence of the streptococci corresponded closely with the maximum presence of *B. coli*. On the six occasions when streptococci were found, in four *B. coli* was present in 0·1 c.c., in one in 1·0 c.c., and in the sixth in 10 c.c. The *B. enteritidis sporogenes* results also corresponded fairly closely with those of the other two indicators.

Markedly different results were obtained by the same observer (Houston, 1902-1903, *b*) in a parallel investigation upon the Tunbridge Wells deep-well waters.

Sixteen samples were examined from four bore-wells. These wells were sunk to a depth of 350 feet, the first 200 feet being through clay, and the remaining 150 feet in the Ashdown sand, a fine-grain sandstone forming part of the Hastings beds.

The total number of organisms developing on gelatine at

20° C. varied from 1 to 17 per c.c., except in one case, when the number was 36 per c.c. In none of the samples were *B. coli*, or allied organisms, isolated from 0·1 and 1·0 c.c., but one doubtful organism was once found in 10 c.c., and three suspicious organisms in 100 c.c. Further examination of these organisms showed that only 1 of them (isolated from 100 c.c. of the sample which gave 36 organisms per c.c.) could be considered a *B. coli*, and this was atypical in respect to one character; the others were allied organisms, but certainly not *B. coli*. As regards *B. enteritidis sporogenes*, this organism was absent in all the samples when 10 and 100 c.c. were examined. Streptococci also were absent in all cases in the amounts (10 c.c.) examined.

These results are in striking contrast with the surface-well figures, and illustrate the marked bacterial purity of uncontaminated deep wells.

These figures may be compared with the results obtained fairly recently by the writer from the examination of a large number of surface wells in and around Colchester. Seventy samples were examined from forty-seven surface wells. The results are shown in the following table:

	Unpolluted.	Doubtful.	Obviously liable to Pollution.	Total.
Number of samples examined ...	9	43	18	70
B. coli present in 0·1 c.c.	0	1	2	3
,, ,, 1·0 c.c.	0	10	9	19
,, ,, 10 c.c.	0	15	11	26
,, ,, 40 c.c.	1	18	13	32
,, absent in 50 c.c.	8	25	5	38
Streptococci present in 1·0 c.c. ...	0	8	4	12
,, ,, 10 c.c. ...	0	11	11	22
,, ,, 40 c.c. ...	0	17	13	30
,, absent in 50 c.c. ...	9	26	5	40

The three groups were determined on topographical grounds alone, the wells being classed as they were inspected

into the three groups, and therefore, before the results of the analyses were known.

The wells classed as 'doubtful' were wells which, when examined, showed no gross evidence of pollution, but which, from their proximity to houses or from being surrounded by manured land, were considered to be probably liable to pollution. All the forty-seven wells were personally examined. Both the *B. coli* and the streptococci results showed marked differences for the three groups of wells (see table). On the whole the streptococci and coli results were in very close agreement. The term *B. coli* was in these investigations rigidly interpreted, and included only *excretal B. coli*, as defined on p. 226. The 37° C. and 20° C. counts showed considerable variability, and these results did not altogether correspond with the topographical classification.

Clark and Gage (Clark and Gage, 1902) record that only one out of 128 samples from 25 tubular wells showed *B. coli*, and that when as much as 100 c.c. of the water were examined. In 432 samples from ponds and lakes, *B. coli* was found only ten times when 1 c.c. was examined; but when 100 and 1,000 c.c. were examined, this organism, with one exception only, was always found. The authors remark that 'the results show with considerable clearness that, in the examination of surface waters from inhabited localities, the question of obtaining positive tests for *B. coli* is only one of taking a sufficient volume of water for the test.'

In 1902 the writer (Savage, 1902, *b*) published the results of an extended investigation, involving many hundred analyses, into the presence and significance of *B. coli* in water-supplies. Unlike most published reports, full topographical descriptions of the different supplies, and also parallel chemical analyses, were added. The waters examined were chiefly from upland surface sources in South Wales.

The examinations made consisted of the enumeration of

organisms developing at 37° C. (agar) and 20° C. (gelatine) in media accurately standardized to a + 1 per cent. reaction, and a quantitative estimation of the number of *B. coli*, 0·5, 2, 10, and 40 c.c. of the water being examined. No streptococci estimations were made.

B. coli was found to be prevalent in these upland surface waters, even from sources of undoubted purity. Even in waters away from all human or sewage pollution, *B. coli* was repeatedly found in 10 c.c., and sometimes in as little as 2 c.c., of the sample. In all such cases, however, sheep were allowed free access to the gathering-grounds, and their excreta were plentiful in the neighbourhood of the waters. The investigations of the writer led him to believe that the origin of these *B. coli* was to be explained as derived in many cases from this animal excreta.

The *B. coli* isolated were not all typical, but 75 per cent. had the following characters: Short bacilli with rounded ends, motile, characteristic growth on gelatine slope without liquefaction, uniform turbidity in broth, fermentation of glucose, production of acid and clot in milk, indol production; 15 per cent. were similar, but did not coagulate milk; and 7 per cent. were similar, but produced no indol. Their ability to ferment lactose was not tested, and the results obtained must therefore be taken as showing that organisms of this class, some of which may not have been true *excretal B. coli*, were prevalent in upland surface waters, and that care is required in forming an opinion from the bacteriological findings alone.*

* In a report published while going through the press, Houston (Houston 1904-1905) records the results of an investigation of certain upland waters. He examined bacteriologically the water of two Highland lochs situated in mountainous, sparsely-populated districts. 'They contain trout in large number, and they are liable to some degree of pollution by the excreta of birds, sheep, deer, and other of the lower animals; but detectable sewage contamination is almost absent.'

Out of twenty-six samples from streams feeding Loch Laggan, *B. coli*

The results of the examination of fourteen samples from eight springs, given in the same report, is in sharp contrast. In only one was *B. coli* or coli-like bacilli found in the whole 50 c.c. examined, and this organism was atypical in that it did not coagulate milk.

Very similar results were obtained with thirteen samples from five water-supplies which consisted mainly of spring water, but into which a certain amount of upland surface water gained access. In only one of the thirteen, and that in 40 c.c., was a typical *B. coli* discovered, while from one other sample (in 10 c.c.) the variety which does not coagulate milk was isolated.

Winslow and Hunnewell (Winslow and Hunnewell, 1902) examined a considerable number of normal waters, all collected in 100 c.c. sterile bottles. They examined 157 samples from presumably unpolluted sources and 50 samples from obviously polluted waters, 48 of them being from streams receiving considerable quantities of sewage and 2 from a grossly polluted shallow well. The results shown in the following table were obtained:

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or coli-like microbes were present in 0·1 c.c. in two samples, in 1 c.c. in sixteen, in 10 c.c. in twenty-five, and in 100 c.c. in all twenty-six samples. Out of eighty-five samples from Loch Laggan itself, similar bacilli were present in 1 c.c. in one, in 10 c.c. in twenty-nine, in 100 c.c. in seventy-one, and absent from 100 c.c. in fourteen. Out of one hundred samples from Loch Ericht, *B. coli* or coli-like bacilli were present in 10 c.c. in one, in 100 c.c. in twenty, and these bacilli were absent in 100 c.c. in eighty.

As regards the attributes of these bacilli, 70 per cent. of those isolated from the feeding streams were flaginac (see p. 225), and 82 and 28 per cent. of those obtained respectively from Lochs Laggan and Ericht were flaginac.

Houston concluded (p. 348) that 'the *B. coli* found in the river, burn, and loch samples were largely not of human origin, but were derived from the drainage of manured land and the excreta of birds and the lower animals. From this point of view the results indicate the danger of too stringent standards and the necessity of interpreting the bacteriological results in relation to the local surroundings.'

These results strikingly confirm those obtained by the writer in 1901-1902, and briefly quoted above.

	Unpolluted Waters.		Polluted Waters.	
	1 c.c.	100 c.c.	1 c.c.	100 c.c.
Samples examined	157	153	50	48
Dextrose broth (positive) ...	40	76	50	31
Lactose plates (positive) ...	13	31	50	26
Colon group	5	11	18	4
Paracolon group	5	5	6	—
B. cloacæ	—	5	1	—
Streptococcus group	3	10	25	22

The methods adopted by these observers, for the 1 c.c. examinations, consisted in adding 1 c.c. of the water to a dextrose (=glucose) broth fermentation tube, incubating at 37° C., and subsequently plating upon litmus-lactose-agar if gas was formed in the tube. For the 100 c.c. results they added about 10 c.c. of phenol dextrose broth (0·25 per cent. phenol) to the 100 c.c. of the sample left in the bottle. After twenty-four hours incubation, 1 c.c. was withdrawn and inoculated into a dextrose fermentation tube. The positive results were plated in the same way. The figures in the table, therefore, mean that, taking the first column, with 157 waters examined in 1 c.c., 40 of them fermented dextrose broth, and 13 of these 40 positive tubes, when examined on lactose plates, yielded *red* colonies. Of these 13 red colonies, 5 on investigation proved to be *B. coli*, while 5 were allied coliform organisms — here called 'paracolon group'—and 3 were streptococci. The paracolon organisms either did not coagulate milk, or the indol results were negative. A similar interpretation is to be placed upon the figures in the other columns.

From the results of the examination of the polluted samples, the authors conclude that the colon bacilli originally present were in many instances killed out and overgrown by the streptococci. Thus, in the last column of the table it will be seen that the dextrose-fermenting

organisms, which the immediate inoculation of the dextrose tube showed to be present in all the 48 samples, disappeared in 44 cases during the two incubations of twenty-four hours each. They concluded that, at least for polluted waters, the use of large samples is not advantageous.

The 1 c.c. results showed a striking difference in the distribution of the sugar-fermenting organisms, and they concluded that 'bacteria capable of growing at the body temperature and fermenting dextrose and lactose are only infrequently present in unpolluted waters.' They also concluded that the group of the streptococci and staphylococci appear to be associated with sewage pollution when present in any numbers.

Petruschky and Pusch (Petruschky and Pusch, 1903) examined a number of waters, and found that the ubiquitous distribution of *B. coli* could in no way be confirmed. In several well waters this organism was not found in the 750 c.c. examined. In forty-five samples of well water the number of *B. coli* varied from 100 per c.c. to nil in 750 c.c. The authors gave no topographical details. Their results confirm those of earlier workers in this country.

The number of *B. coli*, streptococci, etc., in river water must obviously vary enormously with the kind of river, its pollution, etc. The following particulars from investigations upon two English rivers, the Severn and the Thames, will serve as illustrations for this class of water:

In the river Severn, Boyce and others (Boyce, MacConkey, Grünbaum, and Hill, 1902) found that in the neighbourhood of the Vyrnwy River—*i.e.*, from uncontaminated moorland—*B. coli* was absent in 1 c.c., and very few bacteria were present. Immediately above Shrewsbury the number of *B. coli* averaged about 11 per c.c. Between Vyrnwy and Shrewsbury there is a gradual but increasing contamination. Immediately below Shrewsbury, and after the sewage of about 28,000 people had been received, the number of organisms ranged from 7,000 to 20,000 or more per c.c.,

while the number of *B. coli* varied considerably at the different points examined, depending largely upon nearness to the sewer outfall and to the town. From two to four miles away the number varied from 46 to 600 per c.c., and even thirteen miles away 36 *B. coli* per c.c. were met with.

Houston (Houston, 1904, *c*) investigated the Thames as regards the presence of *B. coli* and *B. enteritidis sporogenes* in different parts of the river. The results are roughly summarized in the following table:

Source of the Samples.	Position.	Number of *B. coli* per c.c.	Number of *B. enteritidis sporogenes* per c.c.
Sunbury ...	27 miles above London Bridge	10 to 100	$\frac{1}{10}$ to 1 (usually $\frac{1}{10}$)
Hampton ..	25 miles above London Bridge	10 to 100	$\frac{1}{10}$ to 1 (usually $\frac{1}{10}$)
Barking ...	Opposite sewage outfall	100 to 1,000	1 to 10 (usually 10)
Crossness ...	Opposite sewage outfall	,,	1 to 10 (usually 10)
Purfleet ...	5 miles below Crossness	,,	1 to 10 (usually 10)
Grays ...	10 miles below Crossness	10 to 1,000 (usually 100)	1 to 10 (usually 10)
Mucking ...	20 miles below Crossness	1 to 100 (usually 10)	1 to 10 (usually 1)
Chapman ...	25 miles below Crossness	$\frac{1}{10}$ to 1	less than 1
Barrow Deep	48 miles below Crossness	,,	$\frac{1}{10}$ to 1 (usually 1)

This table shows that the river Thames, twenty-five miles above London Bridge and above the intake of some of the London drinking water, is, as Houston points out, so unsatisfactory from the bacteriological point of view as almost to justify its description as extremely dilute sewage —sewage, for instance, diluted 1,000 to 10,000 times. For these figures, however, the term *B. coli* was not rigidly interpreted, and included all gas-forming, coli-like microbes; so that with a more restricted definition the number of *B. coli* would probably be considerably less.

CHAPTER X

Bacterial Indicators of Pollution

In the early days of water bacteriology, when the specific germs of typhoid fever, cholera, and other water-borne diseases had been but recently isolated and their characters determined, it was not unnaturally hoped that the detection of these organisms in infected water-supplies would become a procedure of practical import and of ready applicability. This hope has not yet been realized, the difficulties of the quest having rendered this line of investigation of but infrequent utility. At the same time, the unreliability of simple enumeration data has been demonstrated, so that, for the bacteriological examination of water to be of practical use, it was soon evident that other methods of investigation must be resorted to. To supply this need the use of bacterial indicators has come into vogue, and has proved of such immense value and reliability that this part of the bacteriological examination of water far eclipses in importance all other branches of the subject.

The object of the bacteriological examination of any water-supply is to ascertain whether that particular water is, or is not, one the consumption of which may give rise to disease or be prejudicial to its users, either at the time of examination or subsequently.

Hygienists are unanimous in recognising that sewage and the excreta of human beings, diseased or healthy, must be looked upon as potential vehicles for disease production. The presence of the excreta of animals must also be looked

upon as prejudicial, since it may contain harmful bacteria and other parasites.

It is clear, therefore, that the detection of the presence of sewage and of human excreta, and to a lesser extent of animal excreta, must be the aim of the water bacteriologist.

A number of organisms have been advocated as fulfilling the requirements necessary for indicators of such contamination. Of these, *B. coli* and allied organisms, *B. enteritidis sporogenes*, and certain streptococci, are the only ones which have been extensively advocated, and merit detailed consideration.

The conditions of a perfect bacterial indicator are fairly definite and obvious. They are:

1. It should be abundant in the substances, for which its presence serves as an indicator.

2. It should be absent, or at least relatively absent, from all other sources.

3. It should be easily isolated and numerically estimated.

4. Its characteristics should be definite and not liable to variation, whereby its distinctive characters might be impaired.

It is nothing to the point whether the organism is itself harmful or not. As an indicator *qua* indicator, this is quite unimportant. If it is pathogenic, this property may give it an added and special meaning of its own as an indicator of particular import—thus, for example, it might point to very recent pollution—but as a general indicator of harmful contamination its pathogenicity is immaterial. Each of the organisms mentioned must now be considered, to see how far it fulfils these conditions of a perfect bacterial indicator.

B. Coli as an Indicator of Pollution.

B. coli is generally accepted as the best indicator of harmful contamination, and is the one most widely used. In view of its importance in this connection, extended consideration is necessary as to how far this use is justi-

fiable. In the chapter on the *B. coli* group it was pointed out that it is not enough to use the term: it must be defined, since organisms included by one observer under this title would be rejected by another.

There are organisms which all authorities recognise as a true *B. coli communis*. There are organisms which are nearly identical, differing only in the absence of one or two minor and variable characters, and there are others which, although allied, yet possess characters, or have not characters, which would make most bacteriologists refrain from classing them as *B. coli*.

In considering the value of this bacillus as an indicator, we must consider how far each of these groups fulfils the conditions of a perfect bacterial indicator.

In regard to the first condition—abundance in the substances for which it serves as an indicator—there is now a considerable body of evidence, and little or no conflict of opinion. For details the chapters on sewage (Chapter IV.) and excreta (Chapter III.) may be consulted. The figures there recorded show that sewage and human excreta, and also the excreta of the ordinary domestic animals, are extremely rich in *B. coli*.

In regard to the second condition—that a perfect indicator should be absent, or at least relatively absent, from all other sources—there is, even at the present time, by no means complete unanimity of opinion. The opponents of the use of this bacterium assert that it is very widely distributed, and is, indeed, found practically everywhere; its detection, therefore, cannot serve as an indicator of anything. It is claimed also that this organism multiplies outside the animal body.

One of the earliest and most important of such objectors was Kruse (Kruse, 1894), who in 1894 pointed out that in general it was not at all clear as to what was understood by the term *Bacterium coli communis*, and that it was less a species than a group of organisms. As usually defined, he

found that this organism was in no way characteristic of the fæces of men or animals, but that such bacteria were present everywhere — in air, in soil, and in water from the most varied sources. Even when the action upon milk and milk-sugar are considered, he stated that micro-organisms with these characters are very widespread. His paper contained no tables of results or experimental data.

Weissenfeld (Weissenfeld, 1900), of the same school, examined fifty-six samples of water, good and bad, testing quantities of 1 c.c. and 1 litre only. He found *B. coli* in all the waters, good and bad alike, and concluded that this organism may be found in waters from any source, contaminated or uncontaminated, if only a sufficient quantity of the water is examined.

The points of identification for his *B. coli* are, however, totally inadequate. He includes as this organism bacilli more or less motile, sometimes non-motile, decolourized by Gram, with vine-leaf surface gelatine colonies, and producing gas in sugar-agar stab cultures. He states that he attaches no importance to milk souring or to indol production, while he does not mention fermentation of the different sugars, apart from gas in sugar-agar stab cultures. Also, his results do furnish some evidence of the value of even these coli-like organisms, since of the thirty waters which he classes as good, in only eight were such organisms found in 1 c.c., while of the twenty-six waters which he classes as bad, these bacteria were present in all but two, in 1 c.c. of the sample.

Refik (Refik, 1896) found colon bacilli constantly present in water of all kinds in the neighbourhood of Constantinople. He distinguished five types, some of which fermented both lactose and glucose, and also coagulated milk, while others gave negative results with all these tests. The only characters in common were motility, classical growth on potato, possession of less than eight flagella, and growth on a special asparagin medium in which the typhoid bacillus

would not grow. Such results are obviously of no value, but the reference is cited here to give an illustration of the kind of evidence frequently adduced to discredit the use of *B. coli* as an indicator of sewage and excretal pollution.

Poujol (Poujol, 1897) examined thirty-four drinking waters, and on twenty-two occasions found *B. coli* when 100 c.c. were examined. The samples were from different sources, such as rivers, wells, and springs. He concluded that fæcal contamination can only exceptionally be invoked as the cause of the presence of *B. coli* in water, and inclined to the view that this organism was widely distributed upon the surface of the earth. He considered that much caution therefore is necessary before condemning a water on account of the presence of *B. coli*, unless it is present in considerable numbers.

The tests he used for the determination of the characters of *B. coli* were extensive, including lactose fermentation, but the paper contains no topographical details as to the sources of the individual samples.

Also, in the only case in which less than 100 c.c. of the sample was examined—*i.e.*, 10 drops—*B. coli* was not found, although present in 100 c.c. Seven of the *B. coli* were tested for virulence, and six were said to be pathogenic.

In another direction evidence has been adduced as throwing discredit on the value of this indicator, in that the organism has been isolated from grain, bread, etc.

Thus Papasotiriu (Papasotiriu, 1902) quotes several German investigators to this effect, and himself examined bread, rye, barley, oats, etc., for *B. coli*, and states that he found the typical organism in all. The characters of the actual organisms isolated are not, however, set out in detail.

He concluded that *B. coli* is constantly present in dough and meal, and very often in cereals, and, apparently from this evidence alone, drew the quite unjustifiable conclusion

that this bacillus is of little or no diagnostic import when found in a water-supply.

Prescott (Prescott, 1903) isolated a number of bacilli from certain corn and meal products which possessed cultural characters similar to those of *B. coli*. Of forty-four cultures, twenty-five were lactic acid bacilli, and nineteen were typical colon bacilli.

He compared their characters with those of twenty-five *B. coli* isolated directly from fæces or sewage. Morphologically, and as regards their fermentative powers and pathogenicity, both groups were identical. The author believes that they are identical in all respects, and that consequently organisms with the same characteristics as *B. coli* are widely distributed in nature, and that their presence, unless they appear in considerable numbers, does not, of necessity, indicate a recent fæcal contamination.

Klein and Houston (Klein and Houston, 1899-1900) also investigated this question.

As regards wheat and oats, although as much as 1 gramme of the cereal was examined, in only two cases were organisms isolated in any way like *B. coli*, and of these one produced only a trace of indol and the other produced none.

As regards rice, flour and oatmeal, when 1 gramme was examined, bacilli which corresponded in all the characters tested to true *B. coli* were isolated from each kind of cereal, but not when a smaller quantity was examined, and only in three out of six samples investigated. The tests used for the identification of this organism were growth in broth, litmus-milk, gelatine shake preparations, characters of the gelatine colonies, formation of indol, and the effects of subcutaneous injection into guinea-pigs. They were all non pathogenic.

Streptococci occurred in more than half the samples of the same substances. Virulent *B. enteritidis sporogenes* were present in a majority of the foodstuffs examined—*i.e.*, in wheat, oats, rice, oatmeal, and wheat-flour. Usually

1 gramme of the cereal had to be examined before these positive results were obtained.

These experiments show that coli-like bacilli are present in many cereals, but that at least a gramme has to be examined to find them. With more rigidly defined tests for *B. coli*, particularly using lactose fermentation, probably some even of these organisms would be excluded. The authors regard the presence of these extraneous micro-organisms as probably due to admixture with surface soil, dust, etc., at the place of growth or during collection and transmission.

Clarke and Gage (Clarke and Gage, 1902) record that a number of samples of various grains were examined for *B. coli*, all of them, however, being carefully collected directly from the fields in which they were growing. In no case were any organisms found which could in any way be confounded with *B. coli*, notwithstanding the fact that twenty-five samples of corn were examined which came from a municipal filtration area to which sewage was being applied daily. As these authors point out, it is hardly a matter for surprise that stored grain should contain a certain number of *B. coli*, in view of the fact that the excreta of mice and rats contain that organism, animals generally abundant in grain ships and storage places.

On the other hand, the careful investigations of a number of other observers do not at all support the views quoted above as to the ubiquity of *B. coli*. The researches carried out by Houston, Chick, the writer, and others, on different soils (for particulars see Chapter V.) show clearly that this organism is absent, or relatively absent, in pure soils. The investigations of many workers upon water-supplies (see Chapter IX.) show that this bacillus is only present in those liable to pollution. Indeed, it is not too much to state that **there is no evidence or observations which have ever shown that B. coli, reasonably defined, is**

present in any numbers in sources which have not been exposed to some form of fæcal contamination.

What, then, do these various investigations show? Two deductions are obvious.

In the first place, the term *B. coli* must be defined within reasonably close limits, with characteristics which agree in their essential features with those of this organism as met with in sewage and excreta. When this is done it is at once apparent that the majority of the investigations showing the indiscriminate presence of *B. coli* have to be excluded from consideration, since they do not deal with *B. coli* only, but include also under that head many other bacilli which cannot in any way claim to be that organism. Conclusions based on such investigations are of no value.

The second deduction is the absolute necessity for considering numerical presence as well as the mere detection of the bacillus. *B. coli* is abundant not only in human excreta, but also in the excreta of many animals, including, probably, all mammals. Obviously, with such an extensive natural habitat, this organism must needs be extensively distributed.

Many general observations, however, and also experiments made specially to test the supposition, show that under ordinary circumstances *B. coli* outside the animal body does not flourish, but gradually dies out. Therefore, while the finding of a *few B. coli* may not indicate more than old and possibly harmless excretal contamination, the finding of *numerous B. coli* points clearly to recent and massive fouling with matters of excremental origin.

It is not unnatural, therefore, to find a *few B. coli* in materials which have at one time or another been exposed to excretal contamination.

When these two points, namely, the necessity for a reasonable definition of *B. coli* and for a consideration of numerical distribution as well as mere presence, are taken into consideration, all the objections raised to this indicator,

on the ground of its alleged universal distribution, fall to the ground. For example, Kruse's objections stated above, and so often quoted, are valid enough in the sense in which they were written, and for the organisms to which they were applied. The mistake is to assume that they are equally applicable to *B. coli* more rigidly defined.

The necessity for a numerical determination of this organism has been repeatedly urged by Houston and many others, including the present writer, while of the earlier workers it is of interest to note that Theobald Smith as long ago as 1893 and Freudenreich in 1895 made this clear. The latter investigator (Freudenreich, 1895) drew attention to the fact that when larger quantities, such as 90 c.c., of the water were examined, he frequently found the organism, and this when absent from 1 c.c. He concluded that we fail to find it only in very good waters, and that it is abundant only in bad waters, and maintained that the mere finding of *B. coli* in a water is not enough to condemn it, its presence in small numbers only not being conclusive evidence of contamination. In such circumstances the local conditions and the number of bacteria are of great importance in his opinion. He used a lactose broth medium for isolation, adding varying quantities of the water to flasks containing this medium.

Intimately bound up with this question of the extra-intestinal distribution of *B. coli* is the question of the viability of this organism outside the animal body, and how far it is capable of multiplication there. For, even granting that the natural home of *B. coli* is the animal intestine, it would be a fatal objection to its use as an indicator of excretal contamination if it could be shown that outside the animal body it can indefinitely maintain itself unchanged, and lead a prolonged saprophytic life. Obviously, in such a case its detection in a water-supply (and even its detection in large numbers, if we grant that it can multiply outside the animal body) cannot indicate much. The only reliable

deduction from such a finding would be that such a water had been at some period, possibly long antecedent, in contact with material which came from the intestines of man or animal. It would give no indication of the extent nor of the date of the fouling, and would furnish no guide as to its immediate origin.

The evidence available is quite adequate to completely confute such a possibility. In regard to the vitality of *B. coli* outside the animal body, investigations show that this organism is in its natural habitat in the animal intestine, or at least in the intestines of most of the higher animals; that there it multiplies and persists, but that directly it gets outside the body it finds itself under conditions not favourable to its continued life. It may persist for considerable periods if the conditions are favourable, but sooner or later it dies out.

The facts definitely ascertained as to its absence, or relative absence, from all soils not recently polluted point strongly in this direction. The direct experiments of Houston on watering soil with sewage, and of the writer with tidal mud (for particulars of both see Chapter V.), show clearly that this organism gradually dies out outside the animal body. The self-purification of rivers from *B. coli* which has gained access to them from sewage illustrates the same thing.

As regards the multiplication of *B. coli* outside the animal body, and apart from its mere persistence, it cannot be stated that this does not occur, since, as we know, this organism readily multiplies in the various artificial media of the laboratory, and this at temperatures quite comparable with those found under natural conditions. Also in milk, extensive multiplication of the *B. coli* added through dirty milking undoubtedly takes place.

Under ordinary conditions, however, such as are met with in connection with the examination of water-supplies, we find no evidence of multiplication (except where fæcal matter

directly gains access to water), but, on the contrary, gradual decrease and extinction.

Turning to the third factor of a perfect indicator (see p. 136), owing to the great amount of attention which has been given to it, the detection and numerical estimation of this organism is now a matter of comparative simplicity.

The last condition of a perfect indicator, that its characters should be definite and not liable to variation, is one which cannot be said to be completely fulfilled by this organism.

As mentioned in the chapter on the *B. coli* group, organisms are frequently met with which differ from the type organism *B. coli communis* in one or more unessential characters, while, also, bacilli are met with differing widely from this typical and true *B. coli*, but linked to it by a whole series of intermediate forms. The question at once arises—what is a *B. coli* in the sense of an organism which acts as indicator of harmful pollution, and what is not?

Closely related is another question—how far are these differences those of different species, how far do they merely result from differences of environment? For it is a quite feasible supposition that *B. coli* perfectly typical in their natural habitat, the intestine, may become atypical and lose some of their minor or even their prominent characteristics under the influence of the comparatively unfavourable environment of soil or water. This is a question of great practical importance in actual routine work. Organisms differing in one or more characters from what may be considered the typical excretal type are frequently isolated from water-supplies, and as a practical point we have to consider whether they indicate pollution equally as well as an organism wholly conforming to the excretal type. Of less practical, but still of much importance, is it to consider whether they are differing species, or whether they are excretal coli modified by environment.

Houston (Houston, 1901-1902, *a*, p. 468), from his experi-

ments upon soil inoculated with sewage, particulars of which are given on p. 60, concluded that his results 'seem to indicate that the somewhat atypical members of the coli group persist longer than the strictly typical, or else that the strictly typical forms lose in process of time some of their positive attributes, or retain them only in diminished degree. The latter is an attractive hypothesis, and my results, both this year and last year, would almost seem to lend countenance to such a tentative supposition; yet it must be remembered that there is no proof forthcoming that the *less* typical forms were not in reality present at the commencement of the investigations, but at that period remained neglected owing to the presence in greater abundance of the more completely typical microbes.'

Jordan (Jordan, 1901, p. 317), dealing with this point, remarks: 'It is quite possible that some colon bacilli may become so disguised by prolonged aquatic life as to be no longer recognisable by the methods used. It must be recalled, however, that it is always possible to recover colon bacilli possessed of typical gas-producing qualities from sewage and from polluted river waters that have been stored for some weeks in glass bottles in the laboratory. In one instance we have found colon bacilli yielding typical gas production in a 1—1,000 dilution of sewage that had been standing in a bottle for forty-two days, and in another case we have found them six months after the sewage had been collected.'

Horrocks (Horrocks, 1903) planted out emulsions of typical *B. coli* in unsterilized pure deep-well waters, in unsterilized tap water, in sterilized sewage, and in sterilized polluted Thames water. The samples were kept in flasks at the laboratory temperature.

The organism was quite unchanged in the deep-well waters when first examined after thirty-one days, but when subsequently examined after nine weeks and three months the indol formation power was feeble as compared with that

of the original culture, the colour change in neutral red was retarded, and the coagulation of milk was slightly delayed.

The same changes were noticed in the bacilli isolated from the tap water after two months, from the sewage after two months, and from the Thames water after three months. Agar emulsions were also planted in various samples of soil and kept at known temperatures. The bacillus was isolated quite unchanged from a rich loam at the end of a month, from a virgin loam at the end of six weeks, and from a virgin sandy loam at the end of sixty days, although for the last the soil became very dry.

Horrocks, therefore, found no evidence that a typical *B. coli* may become converted into an atypical variety by a prolonged sojourn in soil.

The writer (Savage, 1905, *b*), in his investigations upon tidal mud, found no alteration in the characters of the *B. coli* kept in contaminated river mud for over three months.

MacConkey (MacConkey, 1905) inoculated *B. coli communis* into sterile broth contained in the interior of a Pasteur candle, which was suspended in a large jar of tap water, changed from time to time, the whole being kept at room temperature, in the dark, in a cupboard. He found that the characters of the bacillus were unchanged after an exposure of 358 days to what was a changing and unfavourable environment.

Direct experiments, therefore, although neither numerous nor conclusive, give no countenance to the hypothesis that the aberrant coli-like bacilli, so frequently met with in nature, were originally typical excretal *B. coli*, which had lost some of their attributes during their sojourn in an unfavourable environment.

The question can be looked at in other ways. Undoubtedly some degree of inconstancy of character is shown by water bacteria—not stable, but more or less transient—and is due to the influence of environment.

Fuller and Johnson (Fuller and Johnson, 1899) refer to

this transient inconstancy. For the purpose of determining the percentage constancy of the results of prominent diagnostic tests, before and after the employment of their transplantation methods, the authors took twenty cultures, representing eleven different species of water bacteria, and carried them through a series of tests on different media, etc., incubated at 20° C., the results being watched for ten days, the tubes being examined daily.

They found a certain amount of inconstancy, the percentage of positive results for the primary cultures being— for motility, 87; liquefaction of gelatine, 98; fermentation with gas in dextrose broth, 92; nitrate reduction, 90; indol production, 97; milk coagulation, 80.

After their rejuvenation method, 100 per cent. constancy of results was obtained. Their rejuvenation method consisted in 'transferring the organism after isolation from the pure culture on agar, to nutrient broth; from the latter, after three days' incubation at 20° C., to gelatine plates; and from a gelatine plate, after the same period of incubation, back to an agar tube again, from which the conventional media are seeded after the customary three days have elapsed.'

The American Committee on Standard Methods (Report of Committee, 1905, pp. 128-141) gives illustrations of this transient inconstancy of results.

Undoubtedly certain of the characters of *B. coli* do show more variability than others, and a separate consideration of certain of them will be valuable.

Motility. — Many observers describe this bacillus as always showing motility, and if the organism isolated does not exhibit motility it is said not to be a *B. coli*. Not infrequently organisms are met with which are perfectly typical *B. coli*, except that they show no motility. Houston, out of eighty-three bacilli obtained from fæces, all otherwise typical *B. coli*, found eleven that were non-motile when examined from young gelatine cultures. In the writer's

experience, in less favourable media, such as soil or water, the proportion of non-motile to motile is still higher.

Gilbert and Lion (Gilbert and Lion, 1893) divided the varieties of *B. coli* found in the stools of sixty healthy men into two groups: (1) Motile; (2) non-motile—making this a basis of classification.

McWeeney (McWeeney, 1904) records that he has isolated from fæces *B. coli*, otherwise quite typical, except that they showed no motility. He also remarks that he has observed cases where the organisms were motile when grown at 18° to 21° C. and motionless at 37° C., and quotes Stöcklin as having isolated 300 strains of coli from normal fæces, and found 184 to be more or less actively motile, whilst the other 116 were motionless.

The writer (Savage, 1905, *a*) has pointed out that he has frequently been able to show that *B. coli*, non-motile when examined, but otherwise quite typical, can be made to show motility after subcultivation on artificial media. Also occasionally an organism, apparently non-motile by one method of examination, would be distinctly motile by another.

The American Standards Committee recognise this, and remark (p. 85): 'Sometimes even young cultures of the colon bacillus do not show motility, but in these cases the process of rejuvenation will usually restore the normal character.'

It must be accepted that all true *B. coli* are potentially motile in that they possess flagella, but it does not follow that when examined they *exhibit* motility. It follows from this that, as a practical test, a single examination showing absence of motility cannot be considered a reason sufficient to exclude that organism from being accepted as a genuine *B. coli*.

In this connection it is of interest to note that Stephens (Stephens, 1905) has shown the existence of non-flagellate typhoid bacilli, the flagella of which can be restored by passage through animals. This is additional evidence that the exhibition of motility may not be such a fundamental character as is sometimes assumed.

Gelatine Surface Colonies.—It can be shown by direct experiment that these show considerable variation with environment.

The writer (Savage, 1904) experimentally investigated this question. He found that the gelatine surface colonies may undergo extensive variation, even when the organism is kept under such comparatively favourable conditions as in broth at 37° C. In some cases markedly abnormal colonies were obtained.

The source of the organism had a very considerable influence upon the type of colony produced. This is shown in the following table taken from the above paper:

Source of the *B. coli.*	Number of *B. coli* with Colonies.		Percentage of Normal Colonies.
	Normal.	Abnormal.	
Drinking-water	36	9	80
Typhoid stool and sewage	4	0	100
Sea water	7	2	78
Oysters and cockles	2	5	29
Soil	1	6	14
Totals	50	22	69

For practical purposes it will be found that the gelatine colonies of *B. coli* isolated from water are usually typical, yet the fact that they are not typical does not, on that account alone, exclude them from being true *B. coli.*

Indol Production.—This is a character which is admitted by many workers to be somewhat variable.

Peckham (Peckham, 1897) found variations in indol production. She concluded that cultures obtained from river water and other sources where proteid material is sparingly found may become so changed by long deprivation of suitable proteid food that they lose this indol-producing power.

The source from which the organism is obtained is of influence in connection with the production of indol. This is shown in the following table, taken from a paper by the writer (Savage, 1905, *a*), giving results obtained, with *B. coli* isolated, by him:

Source.	Number of *B. coli* examined.	Indol produced.	No Indol produced.	Percentage giving Indol Reaction.
Water-supplies	135	127	8	94
Human excreta (healthy) ...	19	18	1	95
Human excreta (infantile diarrhœa)	10	10	0	100
Animal excreta	18	14	4	78
Sewage	5	5	0	100
Soil	12	12	0	100
Tidal river mud	38	34	4	89
Sea water and tidal river water	13	13	0	100
Shellfish (all but one from cockles)	22	16	6	73
Flies	18	17	1	94
Totals	290	266	24	91·7

In this table are included only those organisms which are typical in their other characters, such as acid production, fermentation of lactose and glucose, coagulation of milk, positive neutral red reaction, and typical growth on gelatine slope without liquefaction of the medium.

This table shows that 8·3 per cent. of the *B. coli*, otherwise typical, produced no indol when isolated, the percentage of positive results being lowest with the bacilli isolated from cockles.

Houston, out of 101 *B. coli* from human fæces, found only two which failed to form indol; but remarks that in the bacteriological examination of soil and water it is common to find coli-like microbes which fail as regards indol production. He apparently holds the view that loss of indol

production means either that the microbe concerned was non-human in origin or had lost its pristine vigour from a saprophytic life.

The writer has frequently, after subcultivation in the laboratory, been able to demonstrate indol production with strains of *B. coli* which, when first isolated, yielded none, and he believes that under unfavourable conditions the power of indol production may be lost, to be subsequently often reacquired if the conditions are more suitable.

On the other hand, not a few experienced investigators hold that indol production is a fundamental and invariable characteristic of this bacillus.

The power to produce the neutral red reaction is also somewhat variable.

The other essential characters of *B. coli*, *e.g.*, the production of acid in litmus-milk or in litmus-whey, the power to coagulate milk, the ability to ferment glucose, lactose, and the carbohydrates generally, are much less liable to variation.

These questions have been considered in detail because they are of great practical importance. In the actual bacteriological examination of waters these abnormal or coli-like bacilli are constantly being found. What interpretation is to be placed upon their presence? Three suppositions are possible as to their origin:

(1) That they are derived from normal typical *B. coli* of fæces, which have become atypical by loss of attribute owing to unfavourable environment.

(2) That they are derived from identical atypical organisms in fæces or sewage, which, owing to greater hardiness and adaptability, have flourished better than the typical varieties, and have thus become relatively more abundant.

(3) That they are true saprophytes, natural to water or soil, and have no excretal origin at all.

It is extremely difficult, with present knowledge probably impossible, to decide as to which of these suppositions is correct. On the one hand are the facts that nearly all the coli-like organisms in fæces are quite typical *B. coli*, that in sewage a good many atypical varieties are present, and that in contaminated water and soil the proportion present is still larger. On the other hand, all the direct experimental evidence at present available from implanting typical *B. coli* in sewage, soil, or water is to the effect that no loss of attributes results, except, possibly, that of indol production.

In this connection the following table recently compiled by Houston,* showing the characters of *B. coli* isolated by him from different sources, is very interesting. For a definition of the term *flaginac*, see p. 225.

Source.	Number of Specimens of *B. coli* or Coli-like Microbes on which Percentage is based.	Percentage Number of 'Flaginac' *B. coli*.
Milk	343	61
Human fæces ...	101	85
Sewage	Several hundreds	65 to 85
Oysters	464	43
Estuarial waters	183	66
Water-cress	81	40
'Washings' of cress ...	52	52
Water in which cress was grown	42	76
London filtered water ...	232	31

From the point of view of the water bacteriologist, the coli group, using the term in its widest sense, includes three subgroups :

1. The true *B. coli* as found in the intestines.

2. Organisms which differ from these excretal coli by the loss of one or more attributes.

3. Allied organisms, widely differing—*e.g.*, by the posses-

* A. C. Houston, 1905, 'The Bacteriological Examination of Milk.' Report to London County Council.

sion of added properties, such as power to ferment starch or to liquefy gelatine.

Subgroup 1 includes both the saccharose and the non-saccharose fermenting varieties, and certainly indicates excretal contamination.

Subgroup 3 includes organisms which are clearly not *B. coli*, and cannot claim to share the significance which attaches to that organism as an indicator of excretal pollution. They doubtless have a significance, but it is independent of that of *B. coli*, and each variety of organism must be considered on its merits.

Subgroup 2 is the one which it is most difficult to appreciate. It includes varieties the significance of which have not been clearly established, and upon the importance of which as pollution indicators there is as yet great diversity of opinion. Under this group would be included organisms only atypical in that they did not produce indol or failed to coagulate milk.

The personal opinion of the writer, as the result of his own experience, and from extended experiments upon the points at issue, is that the key to the solution of the difficulty turns upon the variability of the characters of this organism, and upon which properties are lost in these atypical forms, since all characters are not of equal value. Organisms which differ from the typical excretal type only in the fact that they show no motility or that their gelatine colonies are atypical may be regarded as not having their significance diminished on these grounds. Similarly, the loss of indol-production power would not reduce their significance very markedly.

On the other hand, the absence of glucose or of lactose fermentation would at once, in the writer's opinion, exclude the organism isolated from being considered *B. coli* at all, and the significance of such bacilli as indicators of harmful contamination would be greatly impaired, since the evidence cannot be altogether negatived that either they may not be

excretal in origin, or, if derived from *B. coli* from the intestines, that they have had their characters altered by a saprophytic life so *prolonged* as to take away most of their value as excretal indicators. They may indicate only long antecedent and now harmless contamination.

Undoubtedly this question of possible variation and loss of attributes complicates the subject and detracts from the value of this indicator.

Two widely divergent attitudes have been adopted by bacteriologists. The extremists on one side hold that for any isolated coliform organism to be considered an indicator of sewage and excretal pollution it must have all the attributes of the typical *B. coli communis* of Escherich (with or without saccharose fermentation), and they reject all organisms which are deficient in any one character as not being this organism and as being devoid of significance. At the other extreme are those bacteriologists who include as *B. coli* organisms widely differing from one another, and for some of which there is no reliable evidence that they were derived from the intestine of man or animal.

The latter view includes organisms about whose significance we are in doubt; the former view breaks down in practice.

In actual practical work every bacteriologist of any experience in the examination of water-supplies must have constantly come across samples which contained no typical *B. coli*, as rigidly defined (or at least from which none could be isolated), while they showed numerous organisms which differed from true *B. coli* in one or more characters. If the extreme view is taken, such a water must be returned as free from pollution. Is *one* quite typical *B. coli* in 100 c.c. of more significance as pointing to excretal contamination than *one hundred* atypical *B. coli* in the same amount, organisms which perhaps only differ from the completely typical in that they give no indol, or are non-motile, or possibly do not coagulate milk? Such a position is

obviously untenable, and those who advocate a rigidly defined *B. coli* as the only acceptable indicator of excretal pollution are disregarding actual practical conditions. Also, unless all the colon bacilli present, or a large proportion of them, are isolated, it may well happen that the varieties selected for examination were the aberrant strains, and that if others had been chosen typical organisms would have been obtained. As Houston (Houston, 1902-1903, *a*, p. 548) puts it, 'it seems too little apprehended that even to the expert bacteriologist the picking out of coli-like colonies from gelatine (much more agar) plate cultures is, after all, a *speculative venture*.'

On the other hand, as the definition is widened the value of the determination of these organisms diminishes.

A middle course seems the only justifiable one, and one which offers a practical working basis. The more nearly the organism isolated resembles an excretal *B. coli*, the greater its significance, and the fewer the number required to condemn a water. Stated as a working proposition, the more the characters of the coli-like organisms isolated deviate from that which for convenience may be spoken of as the typical form, the greater the proportionate number of them required to condemn the water. Or, to again quote Houston (*ibid.*, p. 548), 'safety lies in judging contamination by the sum of the characters of the coli present and the proportionate number of such organisms in the sample.'

Summing up these considerations on *B. coli* as an indicator, we arrive at the following conclusions:

1. That *B. coli* is a reliable indicator of *excretal* contamination. It indicates excretal, but not necessarily *human* excretal, contamination.

2. That its value as an indicator of harmful pollution depends both upon the completeness of its attributes as compared with the characteristic organism of human excreta, and upon its numerical presence.

The more nearly its characters are in accord with those

of this typical excretal *B. coli*, the greater its value, and the fewer required to be present to establish evidence of pollution. The further removed its characters are from this typical *B. coli*, the less the significance of its presence, and the greater proportionate number required to establish a prejudicial opinion, until it becomes so divergent that it loses all meaning.

The different characters have not an equal value. Some, such as fermentation of the sugars, liquefaction of gelatine, and acid production in milk, are of so permanent a type that for an isolated organism to show divergence from a typical *B. coli* in any one of these particulars would be to throw grave doubts on its being excretal at all, while others, such as the gelatine surface colonies and the exhibition of motility, are so subject to variability that comparatively little significance can be attached to their, perhaps temporary, absence or modification.

3. That its *numerical* estimation is essential, since, owing to the wide distribution of animals whose excreta contain this organism, its detection in *small* numbers in many water-supplies cannot be taken as evidence of pollution great enough, or recent enough, to warrant those water-supplies being stigmatized as unfit for human consumption; while, on the other hand, its presence in *large* numbers can hardly be due to other than recent and objectionable pollution.

What constitutes large and small numbers in the above sense varies with every class of water, and is dealt with in Chapter XII., on numerical standards.

From the facts and considerations submitted, it will be seen that the limitations to the value of the coli indicator test are three in number :

1. It indicates excretal (including sewage) contamination, but it does not indicate the *source* of that pollution. This is a matter of much importance, since it will be admitted that pollution of a drinking-water supply with

human excreta is a far more serious danger than an infection with animal excreta—for example, sheep excreta—although both may contain an equal number of *B. coli.*

It is not *B. coli* itself which is objectionable, or only to a very limited degree, but that its presence points to pollution with potentially harmful matters—in particular, with possible typhoid bacilli. Sheep pollution is probably comparatively harmless.

This is a distinct drawback to the test, but is one, of course, shared by every other test, chemical or bacteriological, which has been pressed into this service. It points to the necessity of avoiding inflexible standards, and of rationally considering the source of the supply, as well as the bacteriological findings.

2. The indication furnished by this test as to excretal contamination may be reliable enough, but the contamination may be no longer dangerous, since *B. coli* can persist for considerable periods of time in soil and water—certainly longer than the typhoid bacillus.

This, however, is a fault on the right side, and any possible danger of too fine a judgment is removed if careful numerical estimations are made, since if the organism is abundant the pollution must be comparatively recent and therefore dangerous. It is for careful research to say what number of *B. coli* does indicate *recent* pollution.

3. There is the great difficulty which attaches to the precise significance to be given to the bacilli which deviate from the characters ascribed to a true *B. coli communis.*

This is a difficulty to be only completely avoided by the results of prolonged experimental investigation. It is in a fair way to be settled, but certainly, at present, great diversity of opinion prevails.

As has just been pointed out, one of the drawbacks of the coli indicator test is that it does not indicate exactly enough the source of the pollution; in particular, it does not differentiate between a human and an animal origin, while also,

but of minor importance, it does not discriminate between origin from a human intestine that is healthy and one that is diseased.

This limitation has been recognised, and many important investigations have been undertaken to try to discriminate between the different *B. coli* according to their origin and significance.

Such investigations have been made along three different lines:

(*a*) Differentiation by means of different sugars and alcohols.

(*b*) Differentiation by pathogenicity properties.

(*c*) Differentiation by agglutination properties.

(*a*) *Differentiation by Different Sugars and Alcohols.*—It was a not unreasonable supposition that organisms of the coli group derived from animal excreta might possess powers of acting upon the different sugars, cellulose, etc., different from those possessed by similar bacilli from human excreta. As pointed out in Chapter III., investigations have not led to the demonstration of any differences of value, and at present it is not possible to differentiate animal from human origin by any such cultural methods.

(*b*) *Virulence Determinations.*—Undoubted *B. coli* show marked variability as regards their pathogenicity. The question of the virulence of *B. coli* isolated from water has been raised by some workers to a position of much importance, and the testing of the pathogenicity of such bacilli, or of the water itself after incubation with broth, has been advocated as the best criterion of the purity of a drinking-water.

Can it be said that a *B. coli* which is pathogenic to rodents indicates either more recent or more dangerous pollution than one which is not pathogenic? If it could be shown that *B. coli* in sewage and in excreta are highly pathogenic, but lose their virulence in water, then it is clear that the determination of pathogenicity would give highly valuable information as to the recency of the pollution of the water.

If it could be shown that *B. coli* from human sources are more virulent than those derived from animal sources, then, again, the determination would be of value. Lastly, if it could be shown that the *B. coli* in cases of typhoid fever and other intestinal affections are markedly pathogenic, while those from healthy intestines are not, again the determination would serve some (but a very limited) useful purpose.

Blachstein (Blachstein, 1893) advocated the injection of the suspected water after incubation with broth. His paper was, however, most inconclusive.

Levy and Brun (Levy and Brun, 1899) strongly advocated the importance of the virulence test. Their view was that if *genuine B. coli* can be demonstrated in water it is a proof of the fæcal contamination of that water, and such genuine *B. coli* can be distinguished, according to these authors, from unobjectionable coliform bacilli by testing their virulence.

They stated definitely that if 0·5 to 1·0 c.c. of a forty-eight hours' broth culture of *B. coli* derived from normal human fæces be intraperitoneally injected into a guinea-pig, it will kill the animal in one to three days, while in diseased conditions a much higher virulence may be expected. They further stated that virulent coli races gaining access to water will subsist there and maintain their virulence for several weeks, while they had never come across a coli-like form isolated from water capable of killing a guinea-pig in doses of 1 to 2 c.c. when injected intraperitoneally.

The method they suggested was to treat 100 c.c. of the water with 1 per cent. peptone and 1·5 per cent. common salt, and incubate at 37° C. for forty-eight hours. Guinea-pigs are then inoculated with 1 to 2 c.c. intraperitoneally, mice with 0·2 to 0·5 c.c. subcutaneously, and rabbits with 2 to 3 c.c. intravenously. With contaminated waters the animals will die, and virulent *B. coli* be found at the autopsy, with or without other species. It will be noticed that this is not the same thing as the injection of *B. coli* in pure culture, and the fact that virulent *B. coli* are found at the

autopsy cannot be taken as conclusive evidence that they caused the death of the animal, for Beco and many other observers have shown that intestinal *B. coli* can, and do, invade the organs of the body both just before and also after death.

The results of these authors have not been substantiated by other workers.

In the first place, *B. coli* from ordinary human fæces is not usually virulent. Lartigau (Lartigau, 1902) states that general experience abundantly demonstrates that *B. coli* is, on the whole, non-pathogenic as ordinarily found in human fæces, and his paper may be consulted for references to other authors whose results confirm this opinion. He also states, however, that alterations from the conditions normally prevailing in the gut soon increase the virulence of this organism.

Houston (Houston, 1902-1903, *a*) found only 9 out of 101 *B. coli* isolated from healthy fæces to be virulent to rodents. Of these, 4 were from their other characters, probably not true *B. coli*, thus giving only about 5 per cent. virulent. One c.c. of a two days' broth culture, incubated at 37° C., was injected subcutaneously in each case. He found, however, that when broth tubes inoculated with minute quantities of fæces were incubated for two days at 37° C., and then 1 c.c. injected subcutaneously into guinea-pigs, a pathogenic result was almost always obtained, and this although broth cultures of *B. coli* isolated from *parallel amounts* of the same material were commonly either non-pathogenic or, at all events, non-virulent.

Houston (Houston, 1903-1904) reinvestigated this question in the following year, and concluded that the results were due to the presence of a mixture of living microbes probably belonging to the coli group.

The majority of *B. coli* isolated from sewage are also non-pathogenic.

As regards this organism in disease, Sanarelli found that

B. coli isolated from typhoid stools were more virulent than those from healthy stools, and there seems to be a considerable body of opinion that when the intestines are diseased the virulence of *B. coli* is increased.

There is not much information dealing with the virulence of *B. coli* derived from animals, but there is no evidence that its virulence differs from that of the bacillus found in man.

Klecki (Klecki, 1895) found the virulence of *B. coli* from the intestinal contents of a healthy dog to be very variable. *B. coli* from the lower part of the ileum were more virulent than from the colon, and much more virulent than those which were obtained from the parts above the jejunum.

Dealing with actual water investigations, Weissenfeld (Weissenfeld, 1900) examined fifty-six waters, thirty samples supposed to be pure and twenty-six contaminated. He found that the pathogenicity varied independently of the source of the water. The characters accepted by him as sufficient to constitute a *B. coli* were totally inadequate (as pointed out on p. 138), so that these experiments are of very little value in relation to the pathogenicity of true *B. coli*.

Boyce and others (Boyce, MacConkey, Grünbaum, and Hill, 1902) tested a large number of *B. coli* isolated from the polluted river Severn at Shrewsbury. In the majority of cases the organism was not pathogenic when introduced subcutaneously into a guinea-pig.

The writer (Savage, 1903, *a*) conducted a number of inoculations with *B. coli* isolated mainly from water-supplies, very massive doses being injected. The following were some of the results obtained:

Source.	*Virulent to Guinea-pigs.*	*Non-virulent to Guinea-pigs.*
Pure water	1	2
Suspicious water	0	3
Contaminated water	3	6
Sewage or excreta	0	3
	4	14

The distinction between the waters was based upon

several years' intimate knowledge of the particular supplies and upon repeated chemical and bacteriological analysis.

From the experiments there recorded, the writer concluded that they 'lend no support to the view that the pathogenicity of isolated *B. coli* is of help in determining the potency for evil of the water examined.'

Recently Vincent (Vincent, 1905) records that he has isolated a large number of *B. coli* from waters of different kinds, and has tested their virulence by subcutaneous inoculation into guinea-pigs. He found no relation between the degree of contamination of the water and the virulence of the *B. coli* isolated, some polluted waters yielding avirulent bacilli, others virulent, while some pure waters contained virulent *B. coli*.

From these investigations we may conclude that determining the pathogenicity of *B. coli* isolated from water-supplies does not give any additional information either as to the degree of the pollution or as to the dangerousness of the supply as a source of drinking-water.

(*c*) *Differentiation by a Determination of Agglutination Properties.*—Lorrain Smith and Tennant (Smith and Tennant, 1899) found in the water-supply suspected of being causally related to the epidemic of typhoid fever in Belfast in 1898, that many of the races of *B. coli* isolated, when tested with the serum of typhoid patients, showed a well-marked serum reaction, and they suggested that such bacilli possessed a special relationship to the cases of typhoid fever.

Horrocks (Horrocks, 1901, *b*) made a special study of varieties of *B. coli* isolated from typhoid stools, to ascertain if they would show either cultural characteristics or reactions to specific sera, which would enable them to be distinguished from the varieties present in healthy stools.

For the agglutination experiments antityphoid horse serum was used, the dilutions, after admixture with the broth cultures of *B. coli*, being 1 : 50, 1 : 100, 1 : 200, 1 : 500, and 1 : 1,000. Eighty strains of *B. coli* from enteric

stools and seventy from healthy men were examined. The results of the investigation showed that *B. coli* from typhoid stools may be agglutinated by a highly dilute anti-typhoid serum. This sensibility to agglutination was only acquired by environment, and was not truly specific, as in the case of the typhoid bacillus. This was shown by the fact that when the bacilli were removed from their associations, and were preserved for several months on agar slope tubes, the sensibility to the agglutinins in the antityphoid serum gradually declined, and after six months the maximum dilution which caused complete agglutination was only 1 : 100.

Horrocks concluded:

1. That the varieties of *B. coli* in typhoid stools cannot be distinguished by cultural characteristics from the varieties of *B. coli* found in healthy stools.

2. That the varieties of *B. coli* isolated from typhoid dejecta show much greater sensibility to agglutination than the varieties of *B. coli* found in normal stools.

Consequently, he concluded, if cultures of *B. coli* isolated from the suspected water-supplies are found to come within the typhoid range of agglutination, there appears to be fair ground for assuming that the water-supply in question has been polluted with typhoid dejecta.

Houston (Houston, 1902-1903, *a*) tested the agglutination action of the 101 *B. coli* isolated by him from normal fæces, using the blood of a guinea-pig immunized to a slight degree against the typhoid bacillus. When he used a dilution of 1 : 20, he found that ninety-eight gave a negative result, one gave a slight indication of clumping, and two gave strongly positive results. With antityphoid serum from Berne, in a dilution of 1 : 200, very similar results were obtained. Houston concluded that the great majority of the typical *B. coli* derived from the normal stools of healthy persons are not agglutinated with antityphoid serum.

Anticolon serum has not proved of any value. Virulent *B. coli* injected into animals will furnish an anti-colon

serum, but it has not been found possible to obtain a serum which is specific for the colon group, and which would be of use in differentiating true *B. coli* from allied forms, or in supplying information as to the source of the bacillus reacting.

At present, from these interesting experiments one can only draw the deduction that for routine work the information given by agglutination estimations is quite incommensurate with the trouble taken in making them.

CHAPTER XI

Bacterial Indicators of Pollution (*continued*)

Streptococci as Indicators of Pollution.

In Chapter VIII. it was pointed out that the characters of the sewage and fæcal streptococci of Houston are shared by many other streptococci, and that the recent tests of Gordon do not serve to differentiate fæcal streptococci from morphologically similar organisms derived from other sources.

Up to the present there are no characteristics known which enable streptococci derived from sewage or fæces to be readily differentiated from those derived from other sources. In other words, the value of the streptococcus test depends upon the presence of organisms with the morphological characters of streptococci, apart from their cultural characters — that is, upon streptococci *as a class*, and not upon any special varieties. Indeed, when the test was brought forward by Houston in 1899 he advanced the view that streptococci *as a class* could be used as indicators of recent pollution.

In this chapter, therefore, will be considered how far *streptococci as a class* fulfil the four conditions of a perfect bacterial indicator, as defined on p. 136.

1. They are undoubtedly abundant in both sewage and excreta. Details are given in Chapters III. and IV.

2. As regards their absence from all other sources, this cannot be maintained, since they are abundant in saliva, frequent in air, often very numerous in milk, etc.

On the other hand, the substances in which they are present, apart from excreta-polluted materials, are not such as would be likely to gain access to a water-supply. In soils, for example, Houston and the writer have only found streptococci in those quite recently polluted.

Between polluted and uncontaminated waters there is usually a wide difference as regards the streptococci findings.

3. As regards ease of identification and enumeration, if merely the presence of streptococci chains is to be ascertained this is not a difficult matter, but the isolation of streptococci is frequently far from easy, since they often fail to develop upon solid media.

4. As regards variation of characters, if streptococci as a class only are looked for, this question does not arise. There is no evidence available to show how far streptococci from fæces alter their biological characters under adverse environment. The subject has not been investigated.

The value of streptococci as a class, as indicators of excretal contamination, depends then, in the main, upon how far they are absent from sources other than fæces and sewage. With this is intimately bound up the question of the vitality of streptococci outside the animal body.

As regards the vitality of streptococci, two opposite views have been advanced. Houston as long ago as 1898 advanced the view that they were a measure of recent pollution. He remarks (Houston, 1898-1899, *b*, p. 469): 'Streptococci may, as a class, be thought of as germs especially liable to discouragement by unfavourable physical conditions, and, indeed, as surviving only when the conditions are almost ideally propitious. In the present state of our knowledge, therefore, the presence of streptococci in a substance, be it soil, or sewage, or water, suggests recent association of certain ingredients of that substance with an animal host.' He also says, however, that he does not claim that all streptococci are delicate germs, for the results of his own work are against such a view.

Houston has subsequently repeatedly expressed the same opinion. Thus he remarks (Houston, 1902, *d*, p. 144): 'It is not disputed that there may be individual members of the streptococcus class which are comparatively hardy germs, and which may be able to exist and multiply under saprophytic conditions;' but concludes that the results obtained in actual practice show that, as a class, they are delicate germs.

Horrocks (Horrocks, 1901, *a*) isolated certain streptococci, and arrived at the conclusion that if they were found in water in the absence of *B. coli* they would probably indicate pollution of the water-supply by old sewage. He remarks (p. 115): 'In my own work I have repeatedly examined waters which, from their local surroundings, must have been polluted with sewage, and yet I have been unable to find the slightest traces of *B. coli* in them; streptococci were, however, nearly always present.'

Horrocks kept specimens of sewage in the dark at the temperature of an outside veranda, and found that the organisms which most commonly persisted in these old specimens of sewage were varieties of streptococci and staphylococci.

From the results of his experiments upon the inoculation of soil with sewage, Houston (Houston, 1901-1902, *a*, p. 491) concluded that 'the addition of sewage to a soil may be detected by the presence of *streptococci* even in a minimal amount of the soil thus polluted. But the disappearance (relatively, if not actually) of the microbes *unquestionably* to be regarded as *streptococci* seems to be extremely rapid. Nevertheless, the persistence of certain kinds of streptococci, or of pseudo-streptococci, for long periods in the soil was observed on more than one occasion. The differentiation of these streptococci, seemingly of peculiar type from other streptococci of greater significance, . . . is a matter of great difficulty, and involves the personal equation.'

Houston (Houston, 1903-1904) specially tested the vitality

of fæcal streptococci. He tested their vitality in sterilized tap water kept in stoppered bottles in the dark at 20° C. Twenty-five separate strains were tested, in each case 300 c.c. of sterile tap water in a stoppered bottle being inoculated from an agar culture. After thorough mixing, the number of streptococci was determined by making a series of dilutions and inoculating broth tubes with a definite amount from each dilution. The number of streptococci was estimated in the same way each day, the broth cultures containing the different dilutions being incubated for two to three days at 37° C., and then examined microscopically. When 1 c.c. added to broth failed to give a growth on two successive days, the streptococcus was considered to be dead. Under these conditions, 64 per cent. died within ten days, 32 per cent. within four days, while 24 per cent. lived for twenty-five days or longer. The number of streptococci initially added to the water in the different bottles varied from 10,000 to 100,000,000,000 per c.c.

Houston concluded that some fæcal streptococci speedily die when planted in sterilized tap water, but that these cannot be differentiated, for 'it has been found that streptococci yielding the same results to cultural tests do not necessarily yield similar results as regards viability.'

Houston also tested the viability of fæcal streptococci in sterile salt solution (0·5 per cent.). He found that 62 per cent. of the 100 tested survived for at least twenty days.

In regard to these experiments, Houston remarks (p. 527): 'Out of a total of 125 streptococci tested as regards viability, some perished very rapidly, others less rapidly, others, again, only very slowly, and some were still alive at the close of the experiment. Nevertheless, it is impossible to say as yet by cultural tests which streptococci will be hardy, and which easily bereft of life. So that at present in practice it would seem to be desirable to resort (in conjunction with the use of the ordinary cultural tests) to actual tests of viability. It is difficult to say what kind of solution should be employed

for this purpose. Possibly in the case of water samples a portion of the sample should be set aside for sterilization purposes. Then, if streptococci were isolated from the unsterilized water, their viability might be tested for in the same water after sterilization. This would enable the observer to classify streptococci (1) according to their biological characters, and (2) according to their vitality in the water (after sterilization) from which they were originally isolated.'

The experience of the writer as regards streptococci is that certainly most of them are delicate organisms, and rapidly die outside the animal body. He has never found them in soil unless it had been quite recently polluted, while they are also absent from quite pure waters. More resistant forms are sometimes met with. Thus, in polluted tidal river mud (Savage, 1905, *b*) kept in tanks and watered twice a week with fresh but partially sterilized sea water, the majority of the streptococci died out more rapidly than the *B. coli* which were also present, not being found in 1 gramme of the mud after three weeks and four weeks respectively in two of the three experiments. In the third tank streptococci persisted for forty-six days, however, showing that some forms were more resistant.

Taking all the experimental evidence into consideration, streptococci as a class would seem to be naturally parasitic, and unable, in general, to thrive outside the animal body. They are abundant in human and many animal excreta, and in sewage, but are found only in soils and waters which have been polluted.

It would seem that most streptococci die out fairly rapidly under ordinary saprophytic conditions, but that more resistant forms exist and are sometimes met with.

On the whole, it may be concluded that while the presence of numerous streptococci indicates recent pollution, the evidence connecting streptococci with contamination is not so convincing as in the case of *B. coli*. The fact that the

streptococcus group is a very large group of organisms must obviously render it probable that all its varieties are not of equal significance. More investigation is required as to the relative significance of the different kinds of streptococci.

In particular it should be noted that the *absence* of streptococci cannot be said to indicate freedom from pollution, since even in a harmfully polluted water all streptococci may have died out.

To sum up, the presence of streptococci (numerically determined) is a valuable indication of excretal contamination, but their significance has not been established on such a firm basis as that of *B. coli*, so that restraint should be exercised in drawing deductions from their presence. Further, too much stress should not be laid upon their absence.

Bacillus Enteritidis Sporogenes as an Indicator of Pollution.

This organism has also been extensively advocated as a test for sewage and excretal pollution. How far does it conform to the four conditions of a perfect bacterial indicator?

1. It is undoubtedly abundant in both human and animal excreta, while in sewage it is also very prevalent.

2. The second condition is that it should be absent, or at least relatively absent, from all other sources.

From the tables and figures given in Chapter V., it will be seen that both Houston and the writer found this organism more abundant in polluted than in virgin soils, but it was also prevalent in soils not recently contaminated.

Most workers who have studied the distribution of this bacillus have found it widely distributed.

Hewlett (Hewlett, 1899) showed its presence in normal dejecta, and also isolated it from road dust, from laboratory dust, from water, and from eight out of fifteen samples of milk. He concluded that the organism was probably ubiquitous.

Klein and Houston (Klein and Houston, 1899-1900) found virulent *B. enteritidis sporogenes* present in the majority of the foodstuffs examined—*i.e.*, in wheat, oats, rice, oatmeal, and wheat-flour. One gramme of the cereal had usually to be examined before positive results were obtained.

Balfour Stewart (Stewart, 1900) extensively investigated the distribution of this organism. Out of 16 samples of salted fish of which small portions were examined, 6 gave the characteristic changes of *B. enteritidis sporogenes* in milk, and of these 5 caused death when inoculated into guinea-pigs. Nine of the samples were from salted cod.

In 60 samples of grains and seeds, consisting of 20 taken from sample boxes in the sale-room of a wholesale firm, 23 from meals and grains taken from freshly opened sacks in a wholesale warehouse, and 17 samples of flower-seeds from a seed-shop, 41 gave an enteritidis-like growth in the milk, and of these, 30 were fatal to guinea-pigs when inoculated, and 11 were pathogenic, but not fatal. Small portions of the sample were added to the milk in each case.

In 213 samples of milk, about 10 c.c. of each being examined, *B. enteritidis sporogenes*, fatal to guinea-pigs, was found in 18 instances and non-pathogenic in 11 instances, while in 20 it was pathogenic, but not fatal. On 6 occasions this organism was accompanied by *B. coli*; on 11 occasions *B. coli* was found alone.

In 63 samples of shellfish, *B. enteritidis sporogenes* was found alone in 15, and with *B. coli* in 2. *B. coli* was not found alone.

In 70 samples of tinned meat and miscellaneous foods, *B. enteritidis sporogenes* was found alone 8 times; *B. coli* was not found at all.

There was a striking absence of relationship between the presence of these two organisms.

Stewart concluded that dust from the soil is the most probable infecting agent for the grains and for most of the samples of milk.

Glynn (Glynn, 1901) showed that this organism was a normal intestinal inhabitant, and considered it as mainly an index of dust contamination.

As in the case of *B. coli*, it is obviously of fundamental importance to consider the vitality of *B. enteritidis sporogenes* outside the animal body; for, assuming that it has an intestinal origin, if it can exist for long periods outside the animal body, it certainly cannot be used as an indicator of *recent* pollution.

This organism is a spore-bearing bacillus, and, in fact, it is the spores which are used for its detection, and not the bacillus itself. Like other spores, they are highly resistant, and the investigations recorded in Chapter V. show clearly that they can persist for very long periods in soil.

This fact must be kept in view throughout. Its vitality can in no way be compared with that of the typhoid bacillus, and if it is found in a water-supply it is not a reliable deduction that the typhoid bacillus might also be present, or even that it could gain access.

Houston, in his experimental work with soil watered with sewage, found that the subsequent diminution in the number of the spores of this organism was small compared with the reduction in the number of both *B. coli* and streptococci. With animal and human excreta so prevalent, it is not a matter of surprise that such a highly resistant organism should be widely distributed, and found on grain, in dust, etc.

The evidence available shows that it is absent, or relatively absent, from sources which have never been contaminated, but that it is fairly prevalent in sources the pollution of which had taken place even at a long antecedent period.

3. In considering the third condition — that a perfect indicator should be easily isolated and numerically determined—the question arises, whether it is necessary to inoculate animals, or whether the 'enteritidis change,' as

Houston calls it, is sufficient evidence of the presence of this organism.

Houston (Houston, 1904, *b*, p. 213), in regard to this, says: 'In dealing with drinking-waters, especially those not made the subject of routine examination, the advisability of inoculating animals with the whey of changed milk cultures is undoubted. But although a negative result obtained under these circumstances might weaken, it would not destroy belief in the presence of objectionable contamination.'

Since, however, the other bacilli which might be present and cause confusion with *B. enteritidis sporogenes*—in particular, *B. butyricus*—also appear to be inhabitants of sewage and excreta, most bacteriologists content themselves with using the 'enteritidis change' in milk as a sufficient test for the spores of this organism. It must be remembered, however, that by so doing the question of the distribution of these other organisms becomes of importance, and this is one upon which information is far from complete.

There is also another drawback to the applicability of this test. To detect the spores of this organism in water, even if polluted, it is usually necessary to examine large quantities of the water. To examine large amounts—*e.g.*, 500 to 2,000 c.c.—it is necessary to concentrate the germs in the water into a small bulk, and some form of filtering apparatus is required. Such apparatus is troublesome and cumbersome, and the necessity for such a procedure certainly detracts from the value of this organism when used as a pollution indicator.

4. In regard to the last condition—that its characters should be definite and not liable to variation—certain anomalous results are occasionally met with in the milk tubes; but as a rule the results are definite, and in general it may be said that this organism fulfils this condition satisfactorily, and much better than *B. coli*.

Opinions as to the value of *B. enteritidis sporogenes* as an

indicator of pollution are very varied. On the one hand it is strongly recommended by Houston, while Thresh (Thresh, 1904) states that his experience has led him to lay considerable stress upon the test for *B. enteritidis sporogenes* when properly applied.

On the other hand, many bacteriologists find it of but little service, and the English Committee on Standardization of Methods (Report of Committee, 1904) recorded that 'they do not consider that it is essential as a routine procedure to search for the *B. enteritidis sporogenes*, though in certain instances it may be of advantage to do so. A negative result in such cases is probably of more value than a positive one.'

The facts advanced would seem to place a considerable limit to its usefulness. Even if it be granted that its only natural habitat is the intestine, and that its presence elsewhere indicates pollution, yet, in view of the known facts as to the wide contamination of the earth's surface with excreta and *the prolonged powers of resistance of the spores of this organism*, such pollution may be by no means recent, but have taken place at some long antecedent period—a period so remote that all the less resistant pathogenic organisms, against the entrance of which it is needful to guard our water-supplies, would certainly have died out. In other words, the pollution may be so old as to be of no moment. If it is found in a water without *B. coli*, unless it be present in very large numbers, then all that can be claimed for it is that at some period, possibly a very remote period, excrementally-derived material gained access to the water. It does not show that the water is dangerous at the time of the examination, or even potentially dangerous.

If found with *B. coli*, then its presence adds only slight confirmation to the evidence yielded by that organism alone.

If it is not found at all, it is some evidence of purity; but for this to be of any value it must be proved to be absent in a *large* quantity of the water, entailing considerable work,

and far in excess of the value of the results obtained. Even Houston, who has so ably and extensively advocated this test, is constrained to admit (Houston, 1900-1901, *b*, p. 550) that, 'notwithstanding the undoubted value of this test in the bacterioscopic analysis of water, I am inclined to think that a nice judgment is sometimes required in interpreting results.'

CHAPTER XII

Interpretation of Results in the Bacteriological Examination of Water

In the preceding chapters consideration has been given to the numerical presence in waters from different sources of the bacteria developing on ordinary nutrient media and also of the specific bacteria which have been advocated as reliable indicators of contamination. The present chapter is devoted to the difficult problem of determining what bacterial findings are sufficient or necessary to pass an opinion as to the purity, or degree of pollution, of any given water-supply.

Certain primary considerations merit attention.

In the first place, it is absolutely necessary for the bacteriologist to know what kind of water is being submitted to him for bacteriological examination. To expect a chemist to give a decision upon the character of an unknown water, supplied to him without any details, is unreasonable. To expect a bacteriologist to pronounce a trustworthy opinion upon a water of unknown origin is absurd, and is a task which should never be undertaken by him.

It is less unreasonable for the chemist, for the chemical findings throw considerable light upon the source of the water, and he is, therefore, not so dependent upon outside information, while also the interpretation of his results is less delicate. The internal evidence as to the source of a bacteriological sample is trifling.

For a sample about which he knows none of the antecedents, the bacteriologist can report his findings, but should be very cautious in drawing deductions from these as to the purity or otherwise of the source from whence obtained.

The *minimum* facts which should be in the possession of the bacteriologist are:

1. The kind of water—*i.e.*, deep or shallow well, spring, river, etc.

2. The particulars of sampling—*i.e.*, from tap, reservoir, filtered or unfiltered, etc.

3. The particulars of collection—*i.e.*, time of collection, precautions taken in collection and transmission.

Details as to previous rainfall are also useful.

In the next place, it is necessary to consider the deductions which a bacteriological examination of a water-supply is capable of furnishing. The object of all examinations of water-supplies is to determine not only whether the water is actually polluted with matters of excrementitious and harmful nature, but also whether it is liable to pollution in the future. As everyone knows, a water at the time of its examination may be free from actual contamination, and yet be liable to such pollution.

It is not pushing the findings of a bacteriological examination too far to say that under most circumstances the bacteriologist is in a position to say whether a water has been polluted with excrementitious matter or not; and, further, to say whether it is, or is not, at the time of examination, showing evidence of continued pollution. On the other hand, from the results of such an examination it is never possible to say that the water will remain free from excretal contamination. In other words, it is never justifiable to say in a report, from the examination of a single sample, or even from a number of samples, apart from topographical examination, that a water-supply is free from all risk of contamination. Such a report can only state that

there is no evidence of contamination at the time of examination or at a comparatively recent antecedent date.

In this respect the sanitary or topographical examination and the bacteriological examination show considerable differences.

Inspection and careful examination of water-supplies is invaluable, and should never be omitted. Such inspection will frequently show clear evidence of pollution, or, on the other hand, the conditions may be found to be so satisfactory that an opinion as to the purity of the water and its freedom from risk of contamination can be at once given. But in a very large proportion of the cases inspection alone does not enable such a decided opinion in either direction to be given. The conditions found to be present do not show freedom from all risk of contamination, and yet are not sufficient to condemn the water. It is in such cases that bacteriological examinations are essential.

It should also be remembered that the bacteriologist can actually *measure* the *degree* of pollution, whereas the topographist can only indulge in vague expressions of opinion which have no comparative value.

In another direction, also, bacteriological investigations are invaluable, and that is in the *systematic* examination of water-supplies. Such examinations, if made regularly and frequently, enable quite unsuspected sources of pollution to be brought to light, sources which are sometimes by no means readily suspected from ordinary inspection alone.

In other words, the sanitary and the bacteriological examination of water-supplies should never be separated from one another. Careful inspection of all the sources, with bacteriological examination of each separate source of supply, followed by repeated systematic bacteriological examinations, is, in the writer's opinion, the best available method of safeguarding a water-supply, and one which is quite adequate.

With regard to the relative value of the chemical and bacteriological methods of analysis, the writer, from a very large number of samples examined at the same time by both methods, would restate as unchanged his opinion expressed in 1902 (Savage, 1902, *b*, p. 336): 'In general, the chemical figures show much less fluctuation and variation from season to season, and the method appears to be much less sensitive. A water which is contaminated sufficiently to yield evidence of such pollution by chemical analysis will usually show overwhelming evidence pointing to the same conclusion on bacteriological examination; while many waters, on the other hand, show pollution by bacteriological methods which on chemical analysis alone are above suspicion. The bacteriological data require, however, much greater skill and experience to interpret, while the possibility of false deductions from faulty collection or local contamination is very much greater.'

As an interesting illustration and commentary upon the above conclusions, the Bridgend water-supply, as described by Bulstrode (Bulstrode, 1902-1903), may be instanced.

The water-supply of Bridgend was from springs issuing from the carboniferous limestone. The water collected into a natural basin of rock, overflowing by a narrow channel into the river. The suction-pipe of the pump dipped into this channel, and the water was pumped, unfiltered, to supply the town.

The water was examined in December, 1900, three times in 1901, and more than six times in 1902, both chemically and bacteriologically. The bacteriological examinations, which were made by the writer, were in every case unsatisfactory, and the water was repeatedly condemned by him. *B. coli* was abundant, being frequently present in as small an amount as 1 c.c. or less.

On the other hand, the chemical analyses, conducted with samples of water collected at the same time and examined in the same laboratory (by the writer or his assistant), were

satisfactory on every occasion except one, and showed no evidence of pollution.

As part of the Local Government inquiry, fresh samples were submitted to the bacteriologists and chemists of the Board, and the above findings were confirmed.

Bulstrode, by careful investigation, was able to show that the water-supply was liable to contamination by foul matters passing down from the surface to the water level, through the highly fissured limestone, and by the periodical floodings to which the basin wherein this water was collected was exposed. The difference between the chemical and bacteriological results was very marked, and, as Bulstrode remarks, 'it seems, I think, a fair inference from these reports that had chemical analysis alone been relied upon—and, indeed, such analysis is still being relied upon in the vast majority of the water-supplies of this country—the assistance of the Local Government Board would not have been invited, and the water would have continued to be regarded as far beyond suspicion.' It only remains to be added that numerous topographical inspections of the water had been made before Bulstrode's inquiry, and the supply had been pronounced satisfactory and free from contamination.

In interpreting the results of the bacteriological analyses of waters, there are considerable difficulties. Even with full particulars, it is a matter of great difficulty, in a certain number of cases, to give a reliable opinion as to the freedom from contamination of the supply examined. At the extremes of purity and gross pollution there is no difficulty, but for waters of intermediate character judgment is not always easy. Some have attempted to solve the problem by laying down arbitrary standards both as to the number of organisms which may be present, and as to the allowable number of the different indicators of pollution.

It is now generally conceded that *fixed arbitrary standards*

are useless, and this has been the writer's opinion for a number of years.

On the other hand, while arbitrary and inflexible standards are to be avoided, guiding standards of some kind must be, to a certain extent, set up and followed; and, indeed, every working water bacteriologist has some general standards of his own which he follows.

The essential fact to remember is that such standards are not rigid, fixed numerical standards, but rough guides, and liable to modification with varying circumstances. To merely condemn all standards, and to supply nothing in their place, is to leave the inexperienced bacteriologist without means by which to fairly judge samples submitted to him. The following remarks are admittedly very imperfect, but the present state of the subject does not admit of more precise findings or more inflexible standards.

A consideration of the results given in Chapter IX. and elsewhere shows clearly that it is of primary importance to consider each class of water by itself. Standards, for example, suitable for a surface supply are absolutely inapplicable to a spring or deep-well water.

There are five bacteriological estimations commonly or frequently made—namely, the number of organisms developing on gelatine plates, the number of organisms developing at blood heat, the enumeration of *B. coli*, of streptococci, and of *B. enteritidis sporogenes*. Guiding standards must be considered for each of these.

Number of Organisms developing on Gelatine Plates.

The number of these organisms is largely an index of the amount of organic matter, although the results of numerous experiments show that there is no constant, or exact, relationship between the two. Still, the addition of organic matter almost invariably means an addition both of foreign bacteria and of material which enables the water,

for a time at least, to become a better nutrient medium, and so causes an increased proliferation of bacteria.

A low gelatine count is, therefore, a satisfactory feature; but, on the other hand, a high gelatine count cannot, in itself, be considered a sufficient reason for condemning a water. In a deep-well or spring water a high gelatine count is unsatisfactory, since organic matter of any kind should not be found in such sources; but with surface waters the contamination is frequently with harmless organic matter and of comparative unimportance. Thus, for example, the washings of pure upland soil into a mountain stream increase the gelatine count markedly, but it in no way indicate harmful pollution.

Good deep-well and spring waters frequently contain less than 50 bacteria per c.c., developing on gelatine plates, while in surface waters, even when free from pollution, up to 500 or more per c.c. are not infrequently met with.

Number of Organisms developing at Blood Heat.

A great many of the ordinary water bacteria are unable to grow at 37° C., while the organisms associated with excretal and sewage contamination for the most part grow readily at this temperature.

These facts would point to this enumeration being very valuable, but, unfortunately, many harmless soil organisms also grow abundantly at 37° C. The following experiment, carried out by the writer, will serve as an illustration of the limitations of this enumeration:

A pure tap water was collected in a sterile bottle. Agar and gelatine plates were made, and then exactly 1 gramme of a pure soil was added to the water (500 c.c.), and well mixed. Fresh agar and gelatine plates were then at once made. The agar plates were counted after forty hours incubation at 37° C. and the gelatine after three days incubation at 21° C. The following results were obtained (average of three plates for each enumeration):

Water only	...	37° C. count	=	3 per c.c.
,, ,,	...	21° C. ,,	=	76 ,,
Water + soil	...	37° C. ,,	=	1,630 ,,
,, ,,	...	21° C. ,,	=	1,970 ,,

Here the addition of a pure soil (but not virgin soil) which had not been manured or cultivated, certainly, for over three years, and which contained *no B. coli* in 3 grammes, increased the 37° C. count to an extent almost equal to the 21° C. figures.

The blood-heat count is therefore an index of the addition of bacteria other than those natural to pure water, but they need not be harmful; in other words, a high 37° C. count indicates the addition of alien bacilli, but they may be unobjectionable aliens.

The number present in deep-water sources, when pure, is very low, frequently less than 1 per c.c., and 10 or more per c.c. is not satisfactory. In the case of surface waters and rivers, soil washings are common, and a more generous margin (50 to 100 per c.c.) is necessary.

The conditions governing the blood-heat count are, then, somewhat similar to those of the gelatine enumeration. On the whole, a marked increase in the number of bacteria growing at 37° C. is of greater significance than a proportionate increase of the gelatine count.

B. Coli Standards.

Of much greater importance is the interpretation of the *B. coli* estimation. The matter is one of much difficulty, and is complicated by the varying opinions held as to what is to be considered *B. coli*, opinions which complicate both the interpretation of this organism and the data upon which a judgment is based.

The views of different workers show extensive variance. The presence of the typhoid bacillus, even if found in 100 litres only, is sufficient to condemn a water absolutely.

For *B. coli* the position is quite different, since this organism is looked for, not as a pathogenic organism itself potent for evil, but because it serves as a measure of excretal pollution. Obviously, therefore, it is not sufficient to merely ascertain the presence of *B. coli*. It is essential also to have ideas as to the number of them necessary to be present to indicate prejudicial pollution ; in other words, some numerical standards are unavoidable.

Deep-well and spring water should not be liable to any pollution by material containing *B. coli*. Water from these sources, even if originally polluted, must have passed through a considerable depth of soil, and thus have become purified from all evidence of excretal contamination. If such sources are properly protected at their outlets, there is no reason why they should contain any *B. coli*. It is, therefore, justifiable to maintain an attitude of great suspicion towards any water from such sources which contains *B. coli* in 100 c.c. or less. This standard—absence of *B. coli* from 100 c.c.—is based upon a fairly rigid definition of *B. coli*, corresponding to what the writer has called *excretal B. coli* (see p. 226).

In the case of surface supplies and shallow wells the position is very different. For example, considering upland surface waters, the opportunity for contamination by *B. coli* contained in animal (*e.g.*, sheep) excreta may be considerable. The *B. coli* from sheep excreta are indistinguishable from those from sewage or human fæces, yet no one would contend that they are of equal significance, or that it is equally important to prevent their presence.

B. coli is not ubiquitous, but yet is present in probably all mammalian excreta, so that its distribution is extensive. It is these considerations which make the interpretation of the presence of *B. coli* in surface supplies and shallow wells one of much difficulty.

As a matter of experience, on the other hand, it will generally be found that *B. coli*, rigidly defined, are not found

in shallow wells, or in the majority of surface supplies, in 10 c.c. or less, unless that water is being polluted with excrementitious matters in certainly undesirable amount.

Although pollution by animal excreta is generally admitted to be a lesser evil than pollution by sewage or human excreta, yet it is highly undesirable when in appreciable quantity.

While, therefore, admitting that dogmatic standards are especially untrustworthy for these classes of waters, a working standard that the finding of *excretal B. coli* in 10 c.c. or less points to undesirable pollution is both justifiable and in accordance with actual experience. With *B. coli* present in smaller amounts—*e.g.*, 1 c.c. or less—the water can be safely condemned as unsuitable for drinking purposes. If no *B. coli* are present in 50 c.c., the water may probably be safely passed as satisfactory, as far as conditions actually present are concerned.

For rivers used as sources of drinking-water, without artificial purification, similar standards are applicable.

It is a matter of frequent occurrence, however, that typical *B. coli* are not isolated, but organisms lacking one or another of the attributes of the typical *B. coli*. How far are the above standards applicable to these organisms?

It was stated in Chapter X., and it is necessary to restate here, that these organisms cannot be altogether neglected, since they are in the main absent from pure waters, and probably the more closely allied forms were originally derived from excreta. As there explained, in the writer's opinion, the nearer these glucose-fermenting (for of non-glucose-fermenting bacilli our knowledge is so slight that we are not in a position to deduce anything from them) coli-like bacilli approach typical *B. coli* in their characters, the more nearly are our numerical standards for that organism applicable to them, while if they lack essential characters a proportionately greater number must be present to justify an adverse opinion. It is obvious that considerable ex-

erience is required to accurately interpret the significance of these atypical forms.

Streptococci Standards.

Determinations of the number of streptococci have been made much less frequently than in the case of *B. coli;* indeed, many bacteriologists do not consider the enumeration of streptococci of service, so that arbitrary standards are still less applicable.

As a provisional guide, and without attaching an equal significance to the findings, a standard similar to that for *B. coli* may be employed—*i.e.*, their presence in 100 c.c. or less of deep-well or spring water or in 10 c.c. or less of surface and shallow-well waters would justify an adverse opinion as to the purity of the water in question.

Probably a more stringent standard could be adopted, but in view of the incompleteness of our knowledge, the writer would for the present suggest this as a provisional guide.

It is well to remember that on its negative side this test is not of great value, and the absence of streptococci, even in a considerable bulk of water, cannot be taken as showing purity or freedom from danger.

B. Enteritidis Sporogenes Standards.

It is difficult to say what standards, if any, should be used for this organism. According to the data available, it certainly should be absent from 1 litre of deep-well water and from 100 c.c. of surface water.

Summarizing the question of standards, the following, in the writer's opinion, is a convenient rough guide for practical working purposes :

A. Deep Waters

(*Springs and deep wells*).

Gelatine count	Not over 50 per c.c.
Blood-heat count ...	Not over 5 to 10 per c.c.
'Excretal' *B. coli* ...	Should be absent from 100 c.c.
Streptococci	,, ,, ,, ,, ,,
B. enteritidis sporogenes ...	,, ,, ,, ,, 1,000 c.c.

B. Surface Waters

(*E.g., rivers for drinking purposes, shallow wells, upland surface waters*).

Gelatine count	Not over 500 per c.c.
Blood-heat count	Not over 50 per c.c.
'Excretal' *B. coli*	Should be absent from 10 c.c
Streptococci	,, ,, ,, ,, ,,
B. enteritidis sporogenes ...	,, ,, ,, ,, 100 c.c.

It will be noticed that the *surface*-water standards are just ten times as relaxed as those for *deep* waters.

In using these standards, it is essential to remember that they are not of equal significance, and that frequently only some of them will be exceeded, etc.

Thus, the *B. coli* estimation far outweighs in significance all the others, and is *the* essential enumeration upon which to judge the purity of waters. Next in importance is probably the streptococcus estimation, while the gelatine count is the least valuable. It is never safe or justifiable to condemn a water upon the gelatine or blood-heat counts alone.

CHAPTER XIII

Classification of Bacteria found in Water

A VERY large part of the earlier work upon the differentiation of bacteria from water and other sources is rendered quite useless because of the few characteristics investigated, and the undue importance ascribed to characters themselves indefinite and variable, and liable to be differently described by different observers, while in addition the methods of procedure were far from uniform.

At the present day, apart from the half-dozen or so organisms specially looked for, but little importance in the bacteriological examination of water can be attached (with our present limited knowledge) to the isolation and determination of the great majority of the organisms found in water.

It must, however, be admitted that information as to the presence and distribution of other bacteria in nature, even if saprophytic and non-pathogenic, is likely to be of assistance in the bacteriological examination of water-supplies, yielding evidence as to the source of the water, its capacity to support varied bacterial life, etc.

Thus, for example, the presence of considerable numbers of cladothrix (Bismarck-brown cladothrix of Houston) in a water is valuable evidence of recent soil contamination, and in at least two instances the writer has found that its presence has thrown considerable light upon the sources of a water.

The magnitude of the task, and the absence of uniformity of method, has rendered progress in this branch of water

bacteriology extremely slow. The published descriptions are in the main so scanty, rest upon such variable factors, and are so often described without reference to vital accompanying conditions, such as temperature and time, that comparison is hopeless, and it is highly probable that identical organisms are frequently described under different names as distinct bacteria.

Recently, however, decided advances have been made, particularly in America, in regard to more systematic procedure.

Certain principles of procedure are essential.

1. The determining characters should be, as far as possible, those yielding results capable of being recorded by simple positive or negative signs. The descriptions of properties, such as minute morphological features, or the characters of gelatine colonies, are apt to vary greatly with different observers, and cannot form a satisfactory basis of classification.

The American Committee on Standard Methods of Water Analysis (1905, p. 94) remark: 'It is becoming evident that in the future more detailed attention must be given to morphology, and that the biochemical reactions must be made more precise. The hair-splitting methods of differentiation on the basis of arbitrarily selected cultural characteristics which were so common in the earlier days of the science must give way to more rational and broader ideas respecting the distinguishing marks of certain groups of bacteria, and it must also be recognised that these minute organisms are profoundly influenced by environmental conditions.'

2. The descriptions given should always include a definite minimum number of tests, and with, as far as possible, supplementary tests. It is absolutely necessary that the conditions under which the tests are carried out be clearly stated or known. To say, for example, that an organism is non-motile may not be sufficient; it may be apparently non-motile, but be in reality a potentially motile organism,

which, however, did not exhibit motility under the conditions of the particular method of examination selected.

3. Due allowance must be made for the influence of debilitation, caused by an unsuitable environment. Water in particular is a medium which is frequently poor in nutritive material, and therefore likely to exert such an influence. Undoubtedly prolonged growth in water may, to a certain extent, cause a weakened or a delayed development of certain characters which are restored by growth on a more generous medium.

The American Committee (p. 96) give the following data as a *minimum* for the determination of bacterial species, and as suitable for bacteria isolated from water :

1. Source and habitat.
2. Morphological characters.
   Form. Manner of grouping. Dimensions. Staining reactions—(*a*) with watery dyes ; (*b*) by Gram's method. Presence or absence of flagella (motility). Presence of spores and their character. Fission. Capsules. Involution and degeneration forms.
3. Cultural characteristics.
   Mode of growth in and upon : Nutrient broth. Gelatine plates. Gelatine tubes. Agar plates. Agar tubes.
4. Biochemical reactions.
   Action upon milk (reaction and degeneration of casein). Action upon carbohydrates (fermentation, gas formation, production of acidity, etc.). Action upon nitrates. Production of indol. Inhibition of growth by acidity and alkalinity of media. Relation to free oxygen (aërobic and anaërobic growth). Temperature relations (activity of growth at 20° C. and at 37° C., and thermal death-point). Pigment formation. Liquefaction of gelatine.

To exclude the influence of debilitating conditions, Fuller and Johnson's method of rejuvenation (see p. 148) may be used. This method adds greatly to the work, and is, in the opinion of the writer, scarcely necessary unless new species are being described. We now know that certain characters are very little variable, while others, such as indol production, motility, etc., show considerable variability. For routine work it is sufficient to retest all these variable characters, the testing being done after growth on gelatine slope at room temperatures for several weeks, then subcultivating into broth for twenty-four hours, and using the latter for the fresh inoculations.

It is, however, desirable that uniformity of practice should be arrived at in this country.

Of those who have done much to assist in a rational description of bacteria, great credit belongs to Chester (Chester, 1901), who, in his book on 'Determinative Bacteriology,' not only pointed out the necessity of a better system of terminology, but suggested the use of numerous botanical terms for describing their characters with brevity and conciseness.

The characteristics of bacteria fall into three groups, morphological characters, appearance in cultures, and biochemical properties. As regards the respective taxonomic value of these three groups, the ordinary cultural appearances in gelatine, agar, broth, etc., are of least importance, while the biochemical characters are undoubtedly of great value for classification purposes. At the present day the fashion is towards classification into groups rather than a consideration of individual bacteria, and this arrangement is of great assistance.

Such a classification into groups was adopted by Marshall Ward (Ward, 1897). The organisms studied by him were from the river Thames.

Boyce and Hill (Boyce and Hill, 1900) adopted the classification of Ward, and added some extra groups. The

GROUPS.	Source. Number of Cultures.	Bacillus.	Nutrient Agar Tubes. Chromogenesis.	Fluorescence.
Group I. B coli, var. (*a*)	25	+	–	–
,, ,, var. (*b*)	21	+	–	–
Group II. B. lactis aerogenes, var. (*a*) ...	16	+	–	–
,, ,, ,, var. (*b*) ...	11	+	–	–
Groups I. and II. undifferentiated further	29	+	–	
Group III. (1) Proteus vulgaris (type) ...	23	+	±	–
(2) Proteus (varieties) ...	17	+	±	–
(3) B. Cloacæ	21	+	–	–
Group IV. B. enteritidis	6	+	–	–
Group V. Fluorescens liquefaciens ...	33	+	–	+
Group VI. Fluorescens non-liquefaciens	25	+	–	+
Group VII. Subtilis	26	+	–	–
,, Mesentericus vulgatus ...	8	+	–	–
,, ,, fuscus	3	+	brown	–
,, ,, ruber	3	+	rose	–
,, Yellow subtilis	4	+	yellow	–
,, Megatherium	1	+	–	–
,, Non-liquefying	1	+	–	–
Group VIII. Gel. liquef.-Milk acid ...	74	+	–	–
Group IX. Gel. liquef.-Milk alkaline ...	30	+	–	–
		+	–	–
Group X. Gel. not liquef.-Milk acid ...	32	+	–	–
Group XI. Gel. not liquef.-Milk alkaline	29	+	–	–
Group XII. Gel. not liq.-Milk amphoteric	30	+	–	–
Group XIII. Chromogenic bacilli:				
Red	1	+	red	–
	2	+	red	–
	2	+	red	–
Orange	4	+	orange	
	3	+	orange	–
Yellow liquefying ...	6	+	yellow	–
Lact. erythrogenes ...	1	+	yellow	+ red
Group XIV. Chromogenic cocci:				
,, Yellow liquefying	1	–	light yellow	–
	5	–	yellow	–
,, Yellow non-liquefying ...	3	–	yellow	–
,, Pink	5	–	pink	–
Group XV. Non-chromogenic cocci:				
,, Liquefying	8	–	–	–
,, Non-liquefying	27	–	–	–
Group XVI. Sarcinæ:				
,, Yellow	1	–	yellow	–
,, White	2	–	–	–
Group XVII. Streptococci:				
,, Liquefying	1	–	–	–
,, Non-liquefying	3	–	–	

(*To face p.* 193.

groups of these workers were mainly based on morphological and cultural appearances, particularly upon the chromogenic activities, little or no attention being given to biochemical properties.

A much more convenient method of grouping was adopted by Jordan (Jordan, 1903), the groups being largely determined and classified by their biochemical properties. Jordan and his co-workers studied 543 cultures obtained from the Illinois, the Mississippi, and the Missouri Rivers. All the cultures were 'rejuvenated' before being described, and as far as possible the culture media were of uniform composition.

The table from Jordan's paper given opposite shows the essential characters of these groups.

PART II

CHAPTER XIV

Collection and Transmission of Samples

In the collection of samples of water for bacteriological examination great care is required, and the slightest extraneous contamination must be avoided, since small errors in collection may entirely vitiate the results. Precise and seemingly trivial directions must be given, unless the sample is to be collected by an expert.

Samples are most conveniently collected in accurately-fitting glass-stoppered bottles. For an ordinary examination it is sufficient to collect only small quantities—2 to 4 ounces—and the writer always uses 2-ounce bottles, collecting two or more separate samples if a larger amount of water is required. Occasionally it is necessary to examine 1 to 2 litres of water, the water being conveniently collected in a sterilized Winchester quart bottle.

The arrangement figured (Fig. 1) is a convenient and portable form of bottle and tin. The 2-ounce glass-stoppered bottles used are cleaned, and then inverted and dried in the hot-air sterilizer at a low temperature. The stopper is inserted, and the bottle is placed in a tin into which it just slips. The bottom of the tin is covered with several thicknesses of asbestos cardboard, and the lid is similarly lined, so that when in place the bottle is firmly in contact with the asbestos above and below. Cotton-wool can be used instead of, or together with, the asbestos cardboard. The tins with

their contained bottles are then sterilized in the hot-air apparatus (for one hour at 160° C.), and allowed to cool slowly. Labels are placed on the outside of the tins, and they are then ready for use. The tin should not be opened until immediately before the water is collected. The sample should be examined with as little delay as possible. If a delay of more than three hours will occur before the ex-

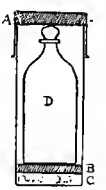

FIG. 1.

A, B, Layers of asbestos cardboard; C, cotton-wool layer.

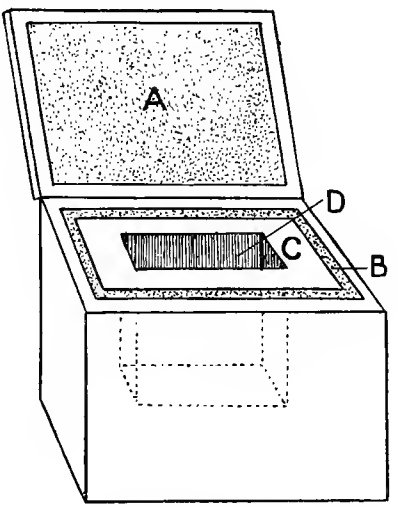

FIG. 2.

A, B, Felt layers lining the box; C, tin box to receive the ice, with depression D for the tins with their contained bottles.

amination, the sample must be at once packed in ice before transmission to the laboratory. Fig. 2 shows a simple form of ice-box. This is made in several sizes to receive one, two, or four tins and bottles similar to those described.

The above is the arrangement used by the writer, and while most workers use their own modifications, there is a general agreement in the use of thoroughly sterilized glass-

stoppered bottles, and packing in ice if delay is unavoidable.*

The collection of samples in small glass tubes or bulbs, with drawn-out necks, broken under the water at the time the sample is collected and subsequently sealed, is much less convenient, and is little used.

These recommendations are in close agreement with those of the English and American Committees on Standard Methods. The English Committee remarks: 'No special precautions beyond those generally recognised are suggested for taking the sample. The samples should be collected in sterile stoppered glass bottles having a minimal capacity of 60 c.c. In special instances it may be desirable to have much larger quantities. Unless examined within *three* hours of collection, the sample must be ice-packed. (The Committee recognise that under all circumstances the sooner the water is examined after collection the more reliable are the results obtained.)'

The American Committee also gives the minimum quantity for bacteriological examination as 2 ounces, while in special cases larger quantities may be required. They remark: 'When bacterial samples are not plated at the time of collection, they shall be kept on ice at a temperature of not more than 15° C., and preferably as low as 10° C.'

For the collection of samples at various depths a number of different forms of apparatus have been devised. One of the simplest and most convenient is that described by Thresh (Thresh, 1904). This is shown in Fig. 3. Its preparation and method of use are described by Dr. Thresh as follows:

'A stoppered bottle of any size can be used, providing the leaden cylinder, partially closed in at the top, will go over it. The glass

* Samples sent by rail must always be in strong locked cases, the bottles being surrounded by plenty of *dry* ice. They should be sent by *passenger* train, and a telegram sent to the laboratory to notify that they are on the way, so that they can be received at the station if necessary.

stopper is removed, and a rubber cork with two perforations inserted in its place. Through one perforation passes a piece of glass tubing about 2 inches long, and through the other a longer piece of tubing reaching to near the bottom of the bottle, and projecting about an inch above the rubber stopper. The projecting tubes are connected by a piece of rubber tubing about 2 inches long. The bottle is suspended

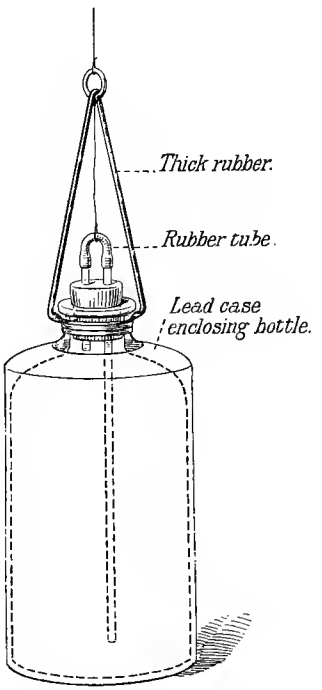

FIG. 3.

by means of a stout band of rubber about 1 foot long, such as is used for door-springs, the free ends being secured tightly to the neck of the bottle by cord or catgut. A metal loop or swivel connects the rubber suspender with the cord or catgut used for lowering the bottle into the water. The loop or swivel is connected with the short piece of rubber tubing uniting the two glass tubes by a piece of string or catgut, of such length that when the bottle is suspended there is no pull upon the rubber tube, which, however, can easily be jerked off when a sharp

pull is given to the suspending cord. The apparatus being arranged, it is lowered to the required depth, a sharp jerk is then given to the suspending cord, when the rubber tube is detached. Water enters through the longer tube and the air is expelled through the shorter tube. Bubbles of air can be seen or heard rising through the water, until the bottle is full, or until only a little compressed air remains in the neck of the bottle. As the apparatus is raised, the air thus imprisoned expands, and prevents water from nearer the surface entering. Catgut serves better than cord for suspending the bottle, and if this is marked off in yards the depth to which the bottle has descended is known. Cord is useless for this purpose. The points requiring chief attention are to see that the rubber stopper is tightly inserted, and that the small piece of rubber tubing can be easily detached by a slight jerk.'

When a special apparatus is not at hand, the sample may be collected in the ordinary collecting bottle after weighting it, by attaching one cord to the neck and a second cord to the previously loosened stopper. The bottle is lowered to the required depth, the stopper is then jerked out by the attached cord, and the bottle fills with water.

When collecting samples from a tap, the water should be allowed to run to waste for five to ten minutes first. When collecting samples from a reservoir, lake, or river, the bottle should be plunged below the surface before removing the stopper, to avoid scum and surface contaminations. It is advisable in such cases to take more than one sample. From a lake or reservoir, samples of the entering and outgoing water should be collected; from a river, samples from the middle and the sides.

The depth at which the sample is collected makes some difference, but under ordinary conditions this is not marked. For the sake of uniformity, it is preferable to take all specimens 1 foot below the surface.

If the sample is to be obtained from a well with an attached pump, it should not be collected until a considerable amount of water has been pumped out; while if a complete investigation is required, a second sample should be obtained after several hours' pumping.

The stopper should only be removed at the actual time of collection, great care being taken that the part of the stopper which goes into the bottle is neither touched nor brought into contact with anything apart from the bottle or water. It must be held by its free end, and at once replaced and screwed in firmly when the sample has been collected. The collected water should not quite fill the bottle.

It is essential that full particulars as to the source of the water be supplied with the sample. It is impossible to give a satisfactory opinion without such information.

The following should be recorded:

1. Date of sampling.
2. Nature of the water—spring, upland surface, etc.
3. Whence obtained—pump, draw-well, river, tap, etc.
4. Precise particulars of sampling—*e.g.*, depth below surface, from middle or sides. If from tap or pump, time during which the water was allowed to run to waste. Filtered or unfiltered.

If from a tap, note if it was directly connected to a main, or if it was connected with a storage cistern or other form of supply.

5. Details as to previous rainfall.

The above particulars should all be supplied to the bacteriologist conducting the examination, and apart from the careful topographical investigation which should be made and recorded in the case of all water-supplies.

The necessity for packing samples in ice if delay has to take place before their examination has been mentioned. It is now well known that bacteria in samples collected in bottles, or other confined spaces, show after a few hours a rapid increase in numbers, and after as short a time as twenty-four hours the increase may be more than a hundred-fold. After a further multiplication for several days a steady diminution takes place. The multiplication is more rapid the higher the air temperature, as met with under ordinary

conditions, while the increase does not affect all the kinds of organisms present alike.

Freudenreich (Freudenreich, 1896) discussed the question whether *B. coli* multiplies in water after collection. He tested the question by two series of experiments. In the first sterile water inoculated with *B. coli*, and in the second natural waters containing this organism, were examined daily. The first series showed that *B. coli* was capable of increase when present alone in a water, but that its increase is not so rapid as that of the ordinary water bacteria. In the natural waters, in most of the experiments, an increase in the number of *B. coli* took place, in some to a very marked extent; but in the comparatively uncontaminated water of the laboratory, in one case there was only a slight increase, and in two a diminution.

Jordan (Jordan, 1901) found that three separate water samples allowed to stand at room temperature showed neither increase nor decrease of *B. coli* after forty-six to forty-eight hours, and remarked that the changes which occur in the numbers of this organism in a water on standing are not very rapid, and are usually in the direction of a diminution. 'It may be fairly assumed that the transportation error is less for the colon determinations than for the colony count.'

When samples of water are packed in ice, it is usually assumed that no alteration in the numbers or kinds of bacteria present takes place, and that a sample examined after, say, twenty-four hours in ice will yield the same results as the freshly collected sample. This cannot, however, be considered an altogether accurate view. In the case of *B. coli* it is probably a close approximation to the truth. Jordan (*ibid.*, p. 301) remarks: 'The hasty assumption that no change in the colon content occurs in ice-packed samples obviously needs justification. Our own experiments are too few in number to warrant generalization, but so far as they go they indicate that no material change occurs in ice-

packed samples within forty-eight hours, a period longer than that usually consumed in transportation.' Summarizing his experiments, made with various natural waters, in five no change took place in the number of *B. coli* after they had been packed in ice for forty-six to forty-eight hours; in one no change occurred after twenty-four hours, but a slight decrease after forty-eight hours; in one there was no change after eight days.

In March and April, 1903, the writer carried out some experiments (unpublished) on the influence of ice storage upon the bacterial content of water. The enumerations were made in all cases upon both gelatine and agar plates, three of each being poured, the agar enumeration being made after forty-three to forty-eight hours at 37° C., and the gelatine after three days' incubation at 20° C. The water samples were kept in 2-ounce glass-stoppered bottles in an ice-box for forty-eight hours (the ice being renewed if necessary), and redeterminations were then made exactly as before, with the same batch of medium, etc., and after very thorough mixing.

Out of four experiments with *B. coli* added to sterilized or natural tap water, in two no alteration took place, in one a very slight reduction (7 per cent.), and in the other a reduction of 17 per cent., for both gelatine and agar plates, took place. The actual figures for this last experiment, conducted with *B. coli* in sterile water, were: Fresh sample, agar 187, gelatine 182; after forty-eight hours in ice, agar 155, gelatine 150.

On the whole, therefore, *B. coli* showed but little alteration after 48 hours' ice-packing, what there was being in the direction of a slight decrease. These results are in accord with Jordan's figures.

Four other experiments made with tap water, either alone or artificially contaminated, gave the results shown in the table on p. 202.

Sample.	Fresh Sample.		After 48 Hours in Ice.		Percentage Alteration.	
	Agar. Per c.c.	Gelatine. Per c.c.	Agar. Per c.c.	Gelatine. Per c.c.	Agar.	Gelatine.
Tap water alone ...	1	240	1	137	0	−43
Tap water + trace of human excreta ...	33	306	10	197	−70	−34
Tap water + sewage	60	8,130	217	7,970	+72	− 2
,, + ,,	11	10,760	5	7,930	−55	−26

These results, even allowing a wide margin for experimental error, show that the ice-packing for forty-eight hours exerted a considerable influence upon the number of organisms per c.c., almost invariably causing a diminution.

CHAPTER XV

General Quantitative Examination

It has been pointed out in Chapter II. that although an enumeration of the number of organisms developing upon solid media in Petri dishes gives neither an accurate estimation of the total number of organisms present, nor precise information as regards antecedent or present pollution, yet in many cases information of value can be derived from such estimations.

The general plan of procedure is the same for all media, but very different results are obtained according to the medium used. Those most used are ordinary nutrient gelatine and nutrient agar, and the method of procedure for these will be described.

Tubes of sterile nutrient gelatine and agar media, about 10 c.c. in each tube, are melted in the water-bath and then cooled down. The agar tubes require the temperature of the water-bath to be raised to boiling-point for complete liquefaction to take place; but the gelatine tubes can be melted, if considered preferable, in the 37° C. incubator. The agar tubes should be cooled down to 41° to 43° C., and maintained at that temperature in the water-bath; but it is better to pour the gelatine tubes at a temperature of about 30° C. In practice, however, it is convenient and sufficient to remove the gelatine tubes from the water-bath (at 41° to 43° C.), or from the 37° C. incubator, at the time the water is to be examined, keeping them at room temperature until inoculated and plated.

The tubes being ready, the sample is well mixed by shaking the bottle many times. As was pointed out in the last chapter, the water should not completely fill the bottle so as to allow of this procedure.

By means of a sterile 1 c.c. pipette, accurately graduated in tenths of a c.c., definite quantities of the sample are transferred to the different gelatine and agar tubes, each tube of agar being replaced in the water-bath directly after the addition. By means of the same pipette the qualitative tests described in the following chapters are also started.

When all the dilutions have been made, the water is distributed through each gelatine or agar tube by rotating the tube between the fingers, and the contents are then at once poured. In doing this the cotton-wool plug is flamed and removed, and the contents poured into a sterile Petri dish, the cover being raised sufficiently to only just admit the top of the test-tube. The Petri dish should have a diameter of not less than 10 centimetres. The plates are then solidified as rapidly as possible. In some laboratories this is done by placing them on wet blotting-paper, in others ice is used. If much water examination work is done, a plate-cooling apparatus is very convenient, and far preferable to the above (see end of chapter). It is important to cool quickly, since it is desirable to have as many surface colonies as possible, while in hot weather gelatine plates left to cool naturally may take a very long time, and multiplication of bacteria and obscuration of colonies result.

When firmly solidified, the gelatine plates should be incubated at 20° to 22° C., and the agar at 37° C.

With an assistant trained to hand the tubes as required to mix, etc., these steps can be done very expeditiously. In some laboratories the procedure recommended by the American Committee on Standard Methods (1905) is used, namely, to pipette the water directly into the Petri dish, and then to add the gelatine or agar, and mix the medium and water thoroughly by tipping the dish backwards and

forwards. This method is not recommended, as the media may easily solidify very rapidly over such a large area, and insufficient admixture and separation of the colonies result. This Committee also remark that 'when agar is used for plating it will be found advantageous to use Petri dishes with porous earthenware covers, in order to avoid the spreading of colonies by the water of condensation.'

These covers were introduced by Hill (Hill, 1905) as a means of preventing the spreading of the colonies on agar plates. They are exactly similar to the ordinary glass cover, except that they are made of porous 'flower-pot' earthenware. The earthenware acts as an absorbent of the condensed moisture. The writer has no personal experience of these covers, but for certain classes of work they should be of value, and preferable to the ordinary method of inverting the plates.

Some bacteriologists, instead of using pipettes graduated as above, use only one mark 1 c.c. pipettes, and the fractions of a c.c. of water are obtained by dilution. For example, 1 c.c. of the sample is removed to 9 c.c. of sterile water, and, after mixing, 1 c.c. of this first dilution will represent 0·1 c.c. of the original sample. In the same way a second 9 c.c. of sterile water can have 1 c.c. of the first dilution added to it, 1 c.c. of this second dilution representing 0·01 c.c. of the original water.

For ordinary routine work, in the opinion of the writer, the use of a 1 c.c. pipette graduated in tenths is preferable, since, while equally accurate, one pipette is sufficient for each water, and the work is carried out more rapidly.

The time of incubation and the method of counting are important, and should be always uniform. The English Committee on Standardization of Methods recommend: 'Counting to be done with the naked eye, preferably in daylight, any doubtful colony being determined with the aid of a lens or low-power objective. *Time of Counting*.—Gelatine plates should be counted at the end of seventy-two hours;

but in all cases the plates should be inspected daily, in order that the count may be made earlier should liquefaction render this necessary. The blood-heat agar plates should be counted at the end of forty to forty-eight hours.'

The American Committee on Standard Methods (1905) advise a somewhat different procedure. They recommend for gelatine plates: 'Incubate the culture for forty-eight hours at a temperature of 20° C. in a dark, well-ventilated incubator where the atmosphere is practically saturated

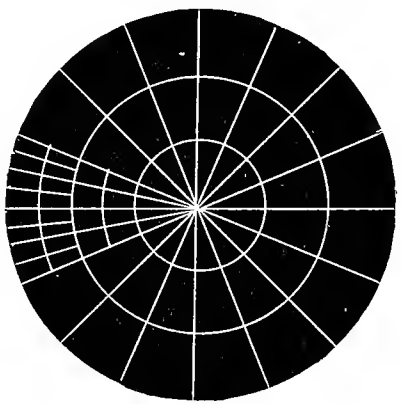

FIG. 4.—PAKES' DISC.

with moisture. After this period of incubation place the Petri dish on a glass plate suitably ruled, and count the colonies with the aid of a lens which magnifies at least five diameters.'

The three days' count method is more in accordance with the practice in this country, and is recommended as preferable.

To count the colonies it is best to count against a dark background, dividing up the area of the plate, to facilitate counting, with lines on the back made with a paraffin pencil. All the colonies on the plate should be counted; but if they are very numerous, and an approximate estimation only is

possible, then, but then only, some mechanical aid such as Pakes' disc may be used, a few segments counted and the total number deduced.

The number of plates which should be poured and the amounts of the sample which should be added to the media tubes will, to a certain extent, vary with the presumed character of the water under examination.

The recommendations of the English Committee are, for an ordinary water, that three gelatine plates be poured, containing respectively 0·2, 0·3, and 0·5 c.c. of the sample, and that two agar plates, containing 0·1 and 1·0 c.c. respectively of the water, be made. These amounts are very convenient.

This Committee also recommends that 'in dealing with an unknown water, and in all cases of doubt, additional sets of plates should be prepared with a dilution of the water (made with sterilized tap water) of ten or hundred fold, according to circumstances.'

As the American Committee points out, the number of colonies upon the plates should not be more than 200 for accurate enumeration.

The terms nutrient gelatine and nutrient agar have been employed. As explained in Chapter II., the exact composition, mode of manufacture, and particularly the reaction of the medium, play an important part in determining the number of colonies which will develop. It cannot be too clearly understood that any enumeration numbers given are not absolute, but only relative, and depend upon the conditions of enumeration. It follows, therefore, that standard conditions are necessary not only for the time of incubation and method of counting, but also for the composition and preparation of the media used.

For nutrient gelatine the English Committee recommend 10 per cent. nutrient gelatine, preferably made with meat (beef) infusion and Witte's peptone, and brought to a reaction of +1 per cent. They recommend that nutrient

agar be prepared with the same constituents as nutrient gelatine, but with the substitution of 1½ per cent. of powdered agar for the gelatine, and with the same + 1 per cent. reaction.

The American Committee (1905) also recommend nutrient gelatine as the standard medium, brought to a + 1 per cent. reaction. The exact methods of preparation are described in their report.

In regard to the choice of meat extract or fresh meat, the writer prefers to use meat extract for water bacteriology work, on account of its convenience, cheapness, and uniformity.

In recording results, the conditions under which they were obtained—*e.g.*, reaction and composition of medium, temperature, and time of incubation, etc.—should also be recorded.

Certain special quantitative enumerations are sometimes made. Thus lactose-litmus agar has been employed instead of nutrient agar, and the number of red colonies only enumerated. Such procedures are not of great value for routine work, since little or no information is derived additional to that obtained from the agar count and the *B. coli* enumeration.

Notes on Apparatus.

1. *A Convenient Water-bath.* — The form of water-bath figured (Figs. 5 and 6) is particularly convenient for plating work, and is little known in this country. A useful size is one with a diameter of 15 centimetres, holding eighteen tubes. By means of the glass tube within the side tube, the height of the water can be adjusted to any desired level. The bath is filled with water, and the media in the tubes melted. The tubes can then be rapidly and conveniently cooled to any desired temperature by running in cold water by means of an indiarubber tube connected to an adjacent water-tap, the excess of water running out of the side tube and the

water adjusting itself to a constant level. The side arrangement also permits the indiarubber tube from the tap to be connected (as shown in Fig. 6), so that a constant level is maintained. This is very useful when the apparatus is used as an ordinary water-bath for evaporation purposes. Copper rings for this purpose are supplied.

By trifling manipulation of the by-pass of a Bunsen burner, it is quite easy to once for all adjust the size of the flame so that when only the by-pass is in use the tempera-

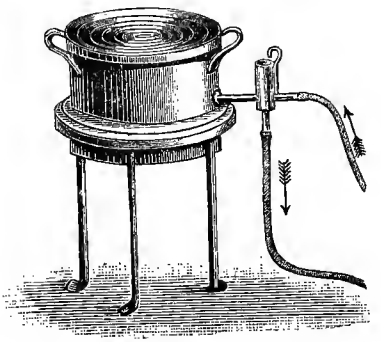

FIG. 5.

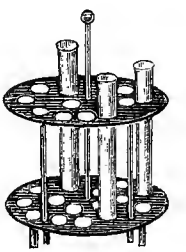

FIG. 6.

ture is maintained within a few degrees of 42° C. for practically an indefinite time.

2. *Sterile Pipettes.*—At least two dozen of these should be provided. One-c.c. pipettes, graduated in tenths of a c.c., and with a total capacity of about $2\frac{1}{2}$ c.c., are convenient. The wide end is plugged with a small piece of cotton-wool, which must not extend beyond the end. A convenient method of sterilization is to place a number in a glass tube, diameter about 1·5 inches, plugged at both ends with cotton-wool, tube and contents being sterilized by hot air. To clean the pipettes after use, boil in very dilute soda-water, wash in distilled water, and dry in the hot air apparatus.

Then plug, place in glass tube, and sterilize. They are then always ready for use when required.

3. *Plate-cooling Apparatus.*—The form figured (Fig. 7), designed by the writer (Savage, 1902, *c*), has been found by him to be very convenient. It consists of a rectangular-shaped copper vessel with a thick plate-glass top, which is

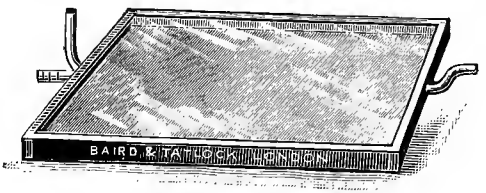

FIG. 7.

firmly fixed to the copper, so that no leakage of water from the interior on to the surface is possible.

The cooling is done by water circulating through the apparatus. This is preferable to the use of ice, since everything is always ready, only a tap requiring to be turned, while the surface is quite level, and no water can get on it.

Plates placed on the glass top have their contained media quickly and uniformly solidified.

CHAPTER XVI

Methods for the Enumeration and Identification of B. Coli and Allied Organisms

As was pointed out in earlier chapters, it is not sufficient to ascertain whether *B. coli* is present or absent. It is necessary to determine also its numerical presence, or absence, in definite quantities of the water.

The enumeration and isolation of *B. coli* involves three processes :

1. The preliminary determination of the probable presence of organisms of the *B. coli* group.

2. The isolation of the *B. coli* organisms in pure culture.

3. The application of determining tests.

Sometimes the first two procedures are combined.

In order to prevent the separate examination and plating of all the different quantities of water examined, it is necessary to have some means by which the tubes which contain *B. coli* can with some measure of probability be distinguished from those which are free from this organism. How far such presumptive tests are in themselves reliable indicators of *B. coli* will be considered at the end of this chapter.

The most important methods which have been used are the following :

Parietti's Method (Parietti, 1890).—In this method, by the addition of phenol and acid, it is sought to inhibit

the growth of organisms other than *B. typhosus* and *B. coli*. Parietti's solution consists of—

Carbolic acid	...	...	5 grammes.
Hydrochloric acid	...	...	4 „
Distilled water	...	...	100 c.c.

A series of 10 c.c. broth tubes is taken, and from 0·1 to 0·3 c.c. of this solution is added to each tube, different amounts being added, so that the broth tubes contain from 0·05 to 0·15 per cent. of carbolic acid. It is convenient to take twelve tubes, and to add 0·1, 0·2, and 0·3 c.c. respectively to four tubes. Small measured quantities of the water are added to these tubes, which are then incubated at 37° C. All the tubes which show turbidity after one to two days' incubation are plated upon ordinary gelatine or carbol-gelatine, and colonies in any way like those of *B. coli* are isolated and identified.

A modification of the above is to use a 5 per cent. carbolic acid solution instead of Parietti's solution, omitting the hydrochloric acid.

This method is now but little used. If the smaller quantities of phenol are added, the other organisms are not inhibited, while with increased phenol weaker strains of *B. coli* may be prevented from growing. The method permits only small quantities of water to be examined, and does not show the numerical distribution.

Phenol broth has been employed by a large number of workers, a frequent method being to add phenol-peptone broth to varying quantities of the water, incubating and isolating the *B. coli*. Péré's method, for instance, recently advocated by Gautié (Gautié, 1905), consists in adding 15 c.c. of ordinary peptone broth and 2 c.c. of a 5 per cent. phenol solution to 100 c.c. of water in a sterile flask, incubating at 37° C., and examining after twelve or more hours for turbidity. If this is present fresh phenol broth tubes are inoculated from the turbid solution; these are incubated, and

the organism subsequently isolated on solid media. Gautié examined not only 100 c.c., but also quantities of the water varying from 100 c.c. to 1 drop.

Growth in Ordinary Broth at High Temperatures.—Vincent (Vincent, 1890) suggested growth at 42° C. after the addition of broth to the sample. He, however, used phenol broth. At 42° C., *B. coli* grows well, but the growth of most of the ordinary water bacteria is inhibited.

Simple incubation of the water with peptone broth at 42° C., or even at 37° C., has been used by a number of workers with satisfactory results. For example, the method employed by Petruschky and Pusch (Petruschky and Pusch, 1903) consisted in adding peptone bouillon to equal quantities of the water in flasks, incubating for twenty-four hours in the blood-temperature incubator, and plating from the flasks which showed uniform turbidity, with the smallest quantity of added water. In these methods no chemical means are used to retard the ordinary water bacteria. The only clue to the presence of *B. coli* is the appearance of turbidity—a very unreliable guide.

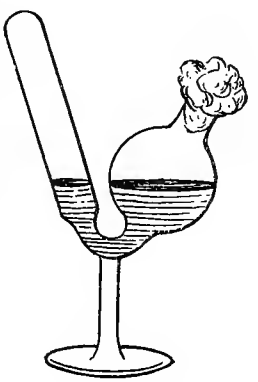

FIG. 8.

Massachusetts Method (Massachusetts State Board of Health, 1898).—Two tests are used. For waters in which *B. coli* is very rarely found a qualitative test is employed, while for waters which generally contain that organism in some numbers a quantitative method is used. The qualitative test consists in adding 1 c.c. of the water to glucose broth in a Smith's fermentation tube (Fig. 8), which is then incubated at 38° C. If after twelve hours gas has collected in the closed arm, the tube is taken out and complete species

tests are made. The absence of gas is considered final evidence that there are no *B. coli* in the water. The broth is then diluted with sterile water, a portion plated on lactose-litmus agar, and the plates incubated at 38° C. for twelve hours. Any red colonies then found are subcultivated upon agar slope tubes, and their characters determined.

In quantitative work, when dealing with waters in which *B. coli* is known to be present, the preliminary test in the fermentation tube is omitted, and 1 c.c. of the water is plated direct upon lactose-litmus agar at 38° C. After twelve hours the red colonies are counted. If few in number, all of them are subcultivated; but if numerous, only a few representative colonies are investigated. The characters are then worked out.

The qualitative test is said to be more delicate, duplicate determinations frequently showing *B. coli* present by the gas test and absent by the plate method.

This method, with slight modifications, may be considered the standard American method. It has the disadvantage of dealing with only small quantities of water, 5 to 10 c.c. at the most, while the absence of any inhibitory chemical occasionally results in the overgrowth of other organisms, and causes difficulty in the isolation of *B. coli*. The first disadvantage is clearly recognised by the American Standard Methods Committee (1905), and they recommend that for appropriate samples 'the fermentation tubes used be of such size as to allow at least 10 c.c. to be tested at a time, and that as many portions be tested as found necessary.' For ordinary water they recommend that 0·1, 1·0, and 10 c.c. be used for the colon test.

Glucose-Formate Broth Method. — Described by Pakes (Pakes, 1900). Various quantities of the water are added to tubes containing glucose-formate broth (broth containing 2 per cent. glucose and 0·4 per cent. sodium formate). The tubes are then incubated anaërobically in Buchner's tubes at 42° C. for two to three days. Gelatine plates are made

from those which show growth. The anaërobic conditions together with growth at 42° C. prevent the development of the majority of the ordinary water bacteria. When dealing with large quantities of water, the organisms are concentrated into a small bulk by filtration through a porcelain tube, and fractions of the concentrated emulsion are then added to the glucose-formate broth and treated as above. This method has not come into general use.

Bile-Salt Broth Method of MacConkey and Hill (MacConkey and Hill, 1901).—The composition of the medium is as follows:

Sodium taurocholate	5 grammes.
Glucose	5 ,,
Peptone	20 ,,
Water	1,000 c.c.

These constituents are heated together until the solids are dissolved. The mixture is filtered, and sufficient neutral litmus solution is added to give a distinct colour. The medium is then distributed into Durham's fermentation tubes and sterilized, by steaming for twenty minutes on three successive days. Glucose was used instead of lactose so as not to exclude the *B. enteritidis* and allied organisms, which are incapable of fermenting lactose.

The absence of the extractives of meat enables the medium to be of an almost uniform composition. The sodium taurocholate prevents the growth of the majority of ordinary water bacteria.

The water is added by pipette to these tubes, which are then incubated at 42° C. A fixed limit of forty-eight hours is taken, since *B. coli*, if present, would give the reaction within that time.

A positive reaction is shown when the medium turns red (due to acid production) and gas is formed in the inner tube. Such a positive result is presumptive evidence of the presence of a member of the *B. coli* group, and the organism is

isolated by plating on one or other of the appropriate solid media. A negative result is reliable evidence of the absence of *B. coli*, but a positive result cannot be taken as being diagnostic of *B. coli*, and actual isolation and cultural identification is necessary.

The chief drawback of the method, as originally described, is that only a few c. c. of the water can be examined at a time for *B. coli*. A convenient modification to obviate this, is to use larger tubes, capable of receiving at least 10 c.c. of the water. In such tubes it is preferable to use bile-salt broth of double strength, so as to keep the proportion of the constituents about the same after the addition of the water.

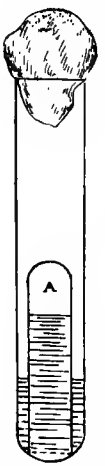

FIG. 9.—DURHAM FERMENTATION TUBE.

This method has been modified by different workers. The most common modification is the incubation of the tubes at 37° C. instead of at 42° C. MacConkey advises the latter temperature as preferable, but almost, if not quite, as good results can be obtained by incubation at 37° C., and the inconvenience and cost of an additional incubator avoided.

Neutral-Red Method.—The use of neutral red as a differentiating agent between *B. typhosus* and *B. coli* is due to Rothberger (Rothberger, 1898). The reaction has been investigated by a number of observers, particularly by Scheffler (Scheffler, 1900) and Hunter (Hunter, 1901). It is best shown in either glucose agar or glucose broth. The typhoid bacillus grown in such a medium does not change it, while *B. coli* converts the red into a yellow colour, with the production of marked fluorescence.

Makgill (Makgill, 1901) experimentally investigated its value for the purpose of isolating *B. coli* from drinking-water, and came to the conclusion that it affords a rapid and

delicate test for the presence of this organism in water; but the writer (Savage, 1901) was the first, in a paper published in the same issue of the journal as Makgill's paper, to describe a method by which neutral red could be used in routine work for the detection of *B. coli* in water.

As pointed out in a subsequent paper (Savage, 1902, *a*), the chief value of neutral red is that it furnishes an indication of which dilutions to examine. The method is similar to that of the simple incubation of broth and water at blood heat, but with the important addition that the neutral red serves as an indicator, showing which dilutions to plate and examine, and prevents the necessity of having to plate all the different dilutions. Neutral red has no selective or retarding action.

In this method different quantities of the water—*e.g.*, 0·1, 0·5, 2·0, 10 c.c.—are added to tubes containing neutral-red glucose broth, while to the remainder in the bottle—about 40 c.c. if 2 ounces are collected—10 c.c. of four times ordinary strength neutral-red broth are added. The incubation is at 37° C., and the tubes are examined daily. The neutral red serves to indicate the tube containing the smallest amount of water which probably contains *B. coli*, as shown by the yellow colour and fluorescence. In every case such tubes must be plated and the *B. coli* isolated; this, as the writer has always pointed out, is necessary, since a positive result does not invariably point to the presence of *B. coli*.

The method is useful, particularly so for upland surface waters; but extended experience has satisfied the writer that, while far preferable to simple broth or phenol media, it is for routine work inferior to bile-salt broth, particularly when dealing with highly contaminated waters. A combination of the two methods is frequently useful.

The neutral-red broth is made as follows: To ordinary broth made from Liebig's extract, peptone, and sodium chloride, and made faintly alkaline to litmus, 0·5 per cent. of glucose and 12 c.c. per litre of a 0·5 per cent. freshly

made watery solution of neutral red (Grubler's) are added. The solution, after preliminary steaming, is filtered into tubes and sterilized for three successive days for thirty minutes each day in the steam sterilizer. A stock of four times strength neutral red broth is also made.

The above methods have the common feature that different quantities of the water are added to liquid media, which on incubation indicate the probability of *B. coli* being present or absent, the organism having subsequently to be isolated. *Direct* plating upon solid media has been used by a number of workers.

Chick (Chick, 1900), for example, used carbol agar, consisting of ordinary agar containing 0·1 per cent. of phenol, distributed into tubes and sterilized by current steam. This percentage of phenol is higher than that recommended by most workers. Direct brushing over carbol gelatine (0·05 per cent.) has been used for many years by Klein, and by Houston. For most of their work the organisms in the water were concentrated by filtration through a porcelain filter, and the filter brushings distributed through 10 c.c. of sterile water. Different quantities were then brushed over phenol-gelatine plates. The plates were incubated at 20° C., and typical, or at least suspicious, coli-like colonies subcultivated and worked out. With highly contaminated waters, 1 c.c. or less was brushed direct over the carbol-gelatine plates.

The direct brushing over lactose-litmus-agar in the Massachusetts method has already been described.

In the examination of moderate and large quantities of water for *B. coli*, two methods of procedure are available. The older method consists in filtering the water through a porcelain filter, and brushing off the deposited organisms into a small quantity of water, thus obtaining the bacteria in a small definite amount of liquid which is treated by one of the above methods. For an extended description

of the procedure see p. 255 *et seq.* This method is tedious and unsatisfactory, and does not alter the relative proportion of *B. coli* to other organisms.

The other, and far preferable, method is to convert the water into a nutrient medium by the addition of nutrient substances such as peptone and meat extract. This is most readily done by adding a sterile concentrated broth. The mixture of broth and water is incubated for twenty-four hours or less, and is then either plated directly on to solid media, or 1 c.c. can be added to bile-salt broth, which may then be incubated and subsequently examined.

In all except the direct plating methods, the colon bacillus has to be subsequently isolated from the liquid media which yield a positive reaction. It is not, of course, necessary to isolate from all the positive tubes, but only from those containing the smallest quantity of water giving a positive reaction.

A considerable number of different solid media have been used for this purpose, of which the best known are nutrient gelatine, bile-salt agar, neutral-red bile-salt agar, phenol gelatine, lactose-litmus agar, and Drigalski-Conradi's nutrose agar. All are serviceable, and with experience of the characteristic colonies good results can be obtained with all.

The colonies on gelatine are as a rule characteristic, but they are not developed in less than forty hours. No special advantage is gained by the addition of carbolic acid. Bile-salt agar was introduced by MacConkey in 1900. A modification of this medium—neutral-red bile-salt agar—suggested by Grünbaum and Hume, is an improvement, and is now used by MacConkey himself.

The three media — lactose-litmus agar, nutrose agar (Drigalski-Conradi medium), and neutral-red bile-salt agar —are all quite satisfactory for the purpose of isolating *B. coli* from water, and can be recommended with confidence.

It must, however, be clearly recognised that these media are only available for isolating *lactose*-fermenting organisms, and should it be desired to ascertain if coli-like bacilli fermenting glucose, *but not lactose*, are also present, then other media must be employed.

For research purposes in particular, it is desirable that such glucose-fermenters should be isolated and studied. For this purpose *glucose*-litmus agar and *glucose* neutral-red bile-salt agar—*i.e.*, the same media, but glucose replacing the lactose—are recommended. A modified method, used frequently by the writer, is to brush the sample for examination over two plates, one of nutrose agar (Drigalski-Conradi medium) and the other of glucose neutral-red bile-salt agar, thus investigating the allied coli-like bacilli, while insuring that lactose-fermenters, if present, shall not be overlooked.

The composition of the medium and the characters of the colonies on the Drigalski-Conradi medium are described in detail on p. 247, in the chapter upon the examination for *B. typhosus*. The other two media are described below.

Lactose-Litmus Agar.—The use of this medium was suggested by Wurtz (Wurtz, 1892). To prepare: Make nutrient agar in the ordinary way, and add 1 per cent. of lactose and sufficient litmus solution just before sterilization. The reaction should be neutral to phenolphthalein. Purified neutral litmus (azolitmin) only should be used, a quantity sufficient to colour the medium a distinct blue being added. After the litmus and lactose have been added the mixture is tubed and sterilized for three days in the steam sterilizer, for fifteen minutes each day. On this medium the lactose-fermenting organisms, including *B. coli*, produce red colonies, due to the formation of lactic acid from decomposition of the lactose, while the non-lactose fermenting bacteria produce blue colonies.

Neutral Red Bile-Salt Agar.—Grünbaum and Hume, who first suggested the use of this medium (Grünbaum and

Hume, 1902), added alkali; but MacConkey prefers it without, the increased alkalinity delaying the appearance of the acid reaction in the case of some organisms.

According to MacConkey (MacConkey, 1905), the medium has the following composition:

Sodium taurocholate (commercial)	5 grammes.
Witte's peptone	20 ,,
Agar	20 ,,
Lactose	10 ,,
1 per cent. neutral red solution ...	5 c.c.
Distilled water	1,000 ,,

It is conveniently made by dissolving the agar in the distilled water in the autoclave, then adding the peptone and taurocholate; the medium is cleared with white of egg in the usual way and filtered. After filtration the lactose and neutral red are added, and the medium is tubed and sterilized for fifteen minutes on two successive days. The neutral red solution should be freshly prepared.

B. coli and other lactose-fermenters grow as red colonies, colour the surrounding medium red, and produce a haze. The surface colonies of *B. cloacæ*, after about twenty hours' incubation at 37° C., are usually raised and opalescent, and some may have a red centre. The colonies of the typhoid bacillus and other non-lactose-fermenting bacilli are white, and colour the surrounding medium an amber or orange tint. As described by Grünbaum and Hume, the medium is made alkaline to the extent of 0·4 c.c. $\frac{N}{0}$ NaOH per cent. beyond the neutral (litmus) point.

With the numerous available methods for estimating the number of *B. coli* in water, the inexperienced bacteriologist may be in doubt as to which to select. In view of the great importance of this estimation, the following considerations are submitted by the writer as some guide until personal experience enables the worker to select a satisfactory

method for himself. Most of the methods given are capable of yielding good results, but some require more experience than others, and it is desirable for the bacteriologist to have one good process with which he is familiar and upon which he can rely.

In considering the different methods, it will probably be conceded that those involving direct plating are decidedly less satisfactory than those including preliminary incubation. The addition of various quantities of the water to a series of tubes of some nutrient medium containing a substance which will pick out the tubes which probably contain *B. coli* is now very generally accepted as the most accurate method by which the numerical distribution of this organism in water can be ascertained. In America the fermentation of glucose with the production of acid and gas is used as this indicating agent. In England the method, perhaps, most frequently used is also the fermentation of glucose, but usually with the addition of an inhibitory agent to the medium (bile salt) to check the growth of other organisms. In the neutral red method the reduction changes in the neutral red serve as such an indicating agent.

The addition of bile-salt is, in the writer's opinion, undoubtedly an advantage, and for all-round work the use of bile-salt broth is the most useful, and this method is to be preferred in preference to any other for the isolation of *B. coli* from water. For quantities of water greater than 10 c.c., this method, unless special apparatus is provided, is not directly available.

The following will be found a convenient procedure: Add 0·1 and 1·0 c.c. of the water respectively to tubes of bile-salt broth. Add 10 c.c. to a large tube of double strength bile-salt broth. To the rest of the sample—about 40 c.c. if a 2-ounce bottle has been used for the collection—add the contents of a tube of four times strength broth, or, preferably, neutral red broth. Incubate all at 37° C. (or 42° C. if an incubator at this temperature is available), and examine

after twenty-four hours' and after forty-eight hours' incubation. If the bile-salt tubes show no gas, *B. coli* can be assumed to be absent in 10 c.c. or less. If acid and gas are produced, the tube giving this positive result with the least quantity of water is further examined and the organism isolated. If no change takes place in the bile-salt agar tubes, the 40 or more c.c. in the bottle is used to inoculate plates of solid media, to see if *B. coli* is present or absent.

In this way it can be definitely ascertained whether *B. coli* is present or absent in 50 c.c. (or 100 c.c. or more, if required), and, if present, in what numbers. For very contaminated waters 0·01 c.c., or other fractions of a c.c., should also be added to bile-salt broth.

For the actual isolation it is sufficient to add one platinum loopful of the medium to a wide tube containing sterile water, and to distribute a little of this over a plate of one of the three solid media indicated as suitable for the purpose. By the next day the colonies will have developed sufficiently for examination and subcultivation. Several colonies—three at least—should be subcultivated, even if they all appear alike. Only one, if found quite conformable in all its characters to a typical excretal *B. coli*, need be fully worked out, but the others should be subcultivated, so that if the one selected is atypical in any of its characters, these can in turn be fully examined.

For spreading the diluted broth over the plates of solid media different devices have been used. Sterile camel's-hair brushes, which were once frequently used, are now rarely employed. They are manifestly unsatisfactory.

In Germany the usual method is to use a glass rod bent at right angles near one end (see Fig. 10). A drop of the diluted broth is placed on the solidified medium in the plate, and distributed by means of the sterilized glass rod. This form of spreader is also largely used in this country. An indiarubber spreader (Fig. 11) has been found very serviceable by the writer. Such spreaders are readily made

by fixing a piece of flat indiarubber, about $\frac{1}{10}$ inch thick and $\frac{1}{2}$ by $\frac{3}{4}$ inch in area, into a wire handle. This is readily done by flattening the end of the wire, making it red hot, and then burning it into the edge of the indiarubber for about $\frac{1}{4}$ inch. The melted indiarubber fixes it firmly when cool. Such brushers can be easily, quickly, and cheaply made. They are placed in wide test-tubes of water, and sterilized in the autoclave, a large number being prepared

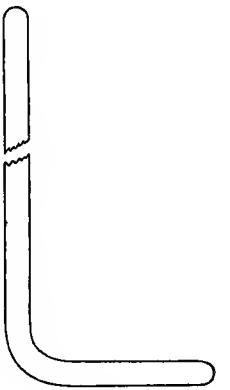

FIG. 10.

FIG. 11.

at one time. They do not scratch the surface of the medium, and are always at hand ready sterilized.

Having isolated the coli-like organism in pure culture, there remains to be considered the necessary identification tests to apply. The characters of *B. coli communis* and allied organisms are fully discussed in Chapter VI.; here it is only necessary to consider the *minimum* tests to be carried out in routine work. The greater the number of tests, the more valuable the data available for the classification of any bacillus isolated; but it is obvious that for ordinary routine

work multiplication of tests takes much time, and in an extended form is impracticable.

The English Committee on Standardization of Methods (1904) recommended the following six subcultivations as a minimum: Surface agar at 37° C., stab and surface cultures in gelatine, litmus-milk, glucose litmus medium, lactose litmus medium, and peptone-water.

The diagnostic characters of the American Committee have already been stated. They involve examination for morphology and motility, growth in gelatine stab, litmus-milk, dextrose broth, peptone-water, and nitrate solution.

Houston (Houston, 1906, *a*) has recently adopted a 'flaginac' basis of classification for *B. coli*. To express the results of subcultural tests of the coli-like microbes isolated, the word *flaginac* is used by him in the following sense:

fl indicates greenish fluorescence in neutral-red broth cultures.

ag indicates acid and gas in lactose-peptone cultures.

in indicates indol formation in broth cultures.

ac indicates acidity and clotting of litmus-milk.

'The word "flaginac" thus indicates that a microbe was indistinguishable, as regards the tests employed, from the typical *B. coli* of the human intestine. When the letters are placed in brackets, an incomplete reaction is indicated. The absence of a character is expressed by the omission of the letters chosen to indicate that attribute.'

The fermentation of glucose and the growth on gelatine are also invariably investigated by Houston.

As the writer (Savage, 1905, *a*) pointed out in a recent paper, the tests of the English Committee can be reduced without detriment to five by the omission of the growth on agar slope, and the following would include all that are essential, and are recommended for routine work.

Growth—

(*a*) On gelatine slope.

(*b*) In Litmus-milk at 37° C.

(*c*) In lactose-peptone litmus solution (in a Durham's tube).

(*d*) In peptone-water.

(*e*) In glucose neutral-red agar shake preparation, or glucose neutral-red broth in a Durham tube, which the writer now finds preferable.

From these the following results should be recorded:

1. Morphology and motility. This can be conveniently investigated from the gelatine slope growth, twenty to twenty-four hours old, and examined as a hanging-drop preparation in sterile broth.

2. Appearance on gelatine slope after two days' and after two weeks' growth. To ascertain characters of growth and also to see if liquefaction takes place.

3. Acid production in litmus-milk, and the permanency of this acidity.

4. Presence or absence of coagulation of the milk. The inoculated milk must be incubated for at least two weeks before a negative result is recorded.

5. Lactose fermentation; production of acid and gas, one or both.

6. Glucose fermentation; production of acid and gas, one or both.

7. Presence or absence of neutral-red reaction.

8. Production of indol. Tested for after five to seven days' growth at 37° C. in peptone-water.

All these results should be recorded as the *minimum* for routine work. They involve the use of only five tubes of media.

The use of the term *excretal B. coli* was suggested in this paper as a convenient one to be used for an organism reacting to all these five tests in the way described in Chapter VI. as characteristic of this bacillus (whether exhibiting motility or not). That is, the use of the term *excretal B. coli* would imply without further description that all these characters were present. These characters

are certainly those possessed by the vast majority of *B. coli* isolated from excreta, and the term is a more convenient one than *typical*, since to the latter so many different interpretations have been given.

The term (without modification) should only be used when these definite characters have been tested for and shown to be present. If any characters are negative, the fact can be mentioned in brackets. Thus an organism having all these attributes, except that it gives no indol reaction, can be readily written *excretal B. coli* (indol −).

In actual practice, if only lactose-fermenters are being considered, the simplest plan is to subcultivate all the colonies under investigation into tubes of lactose peptone solution. Those which show acid and gas production within two days are at once subcultivated into the other four media. If no lactose fermentation takes place within two days, it is not necessary to make further subcultivations. On the other hand, if the basis of investigation is wider, and all glucose-fermenters are being worked out, whether they ferment lactose or not, then all suspicious colonies should be inoculated direct into the glucose neutral-red broth (in Durham's tubes); and if within two days gas develops, with or without a neutral-red reaction, the other four media should at once be subcultivated from this medium. If no glucose fermentation takes place within two to three days, further cultivations are unnecessary. In this way regularity of procedure is insured with a minimum of trouble.

For research purposes it may be necessary to make a greatly extended series of cultural tests, but for routine water examination the above is sufficient.

How far an organism may fail to respond to any of the above tests, and still be considered a *B. coli*, is exhaustively considered in Chapter X.

In recording results simplicity should be aimed at, and + and − signs used as far as possible. The following description of an isolated organism will serve as an

example, the minimum number of tests recommended being used:

Morphology: Coli-like; motility +.

Gelatine slope: Bluish translucent growth; *l* – (fourteen days).

Litmus-milk: *ac* +; coagulation + (two days).

Lactose: + (+ means acid and gas; if acid only, write *ac*).

Glucose: +

Neutral-red reaction: – (*i.e.*, negative response).

Indol: – (*i.e.*, negative response).

Or if the term *excretal B. coli* be accepted, it could be written:

Excretal B. coli (neutral-red and indol –) present.

Presumptive Tests for B. Coli and Excretal Contamination.

The isolation of *B. coli* and other excretal organisms takes time and labour, and many bacteriologists have therefore sought for means whereby their presence might, with considerable probability, be deduced without actually isolating them. Stated another way, they have tried to find bacterial tests, comparatively simple and easy to carry out, which in themselves would indicate whether the water under examination was of good quality or of a suspicious character as regards pollution. Such short-cuts to a definite opinion have been used to a certain extent in England, and more extensively in America, and are generally spoken of as 'presumptive tests.'

In America the production of gas in a Smith's fermentation tube containing dextrose* broth, with a gas formula of $H : CO_2 = 2 : 1$, has been used as, in itself and without isolation of the gas-producing organism, a rough but reliable indicator of the presence of excretal *B. coli*.

* Grape sugar is generally spoken of by American bacteriologists as *dextrose*, while in this country the term *glucose* is preferred to designate the same substance.

It cannot, however, be affirmed that the presence of fermentative changes in the dextrose fermentation tube, even with this definite gas formula, always coincides with the presence of *B. coli*. Winslow and Hunnewell (Winslow and Hunnewell, 1902), for example, found that 40 out of 157 samples of water from unpolluted sources, examined in amounts of 1 c.c., fermented glucose, but only 5 yielded on isolation bacilli of the colon group—a percentage of 12·5. Of 50 samples from polluted waters examined in amounts of 1 c.c., all 50 fermented glucose in the fermentation tube, but only 18 bacilli of the colon group were isolated—a percentage of 36. In some of these cases, no doubt, *B. coli* was present, but had died out; in others the discrepancy is to be ascribed to the fermentation not being associated with *B. coli* at all.

In the same way Winslow and Nibecker (Winslow and Nibecker, 1903) have used the formation of red colonies upon litmus-lactose agar plates as a guide to the purity of the water, a pure water giving few, if any, red colonies, a contaminated water giving a considerable number. This is undoubtedly true in general, and such plates may be of value, but the red colonies (lactose-fermenters) are not all *B. coli*, and their number is not a direct measure of the number of *B. coli*.

Whipple (Whipple, 1903) strongly advocates the use of presumptive tests for *B. coli* on the grounds of the reliability of the deduction, the readiness and ease of application, and the rapidity with which an opinion can be arrived at. His presumptive test is the changes in a Smith's fermentation tube, including the gas ratio. He admits, however, that the amount of gas and the percentage of CO_2 are subject to variation even with pure cultures of *B. coli*, and that a certain amount of error is introduced when the results are taken as actually indicating *B. coli*, without further isolation.

In regard to this particular presumptive test, Fuller and

Ferguson (Fuller and Ferguson, 1905) have recently called attention to its unreliability, and remark that they repeatedly found *B. coli* to be present in fermentation tubes in which the amount of gas was less than 20 or even 10 per cent. after forty-eight hours' incubation at 37° C.

The bile-salt broth of MacConkey and Hill is in itself a presumptive test of value. Its introducers considered that if acid and gas were produced, in the majority of instances they were due to organisms of an intestinal type. Most workers, however, would not consider that taken alone gas and acid formation in this medium is sufficient evidence of pollution, without subsequent confirmation by isolation of the intestinal organism.

The following tables compiled from recent reports by Houston (Houston, 1906, *b*) are of interest in this connection:

FILTERED THAMES WATER.

Time of Year.	Total Number of Samples examined.	10 c.c. of Water examined.			1 c.c. of Water examined.		
		Numbers giving Positive Results with:			Numbers giving Positive Results with:		
		Presumptive.	Confirmatory.	Flagellac.	Presumptive.	Confirmatory.	Flagellac.
January, 1906 ...	436	78	75	56	31	29	17
February, ,, ...	395	94	89	56	31	31	17
March, ,, ...	433	56	54	28	23	22	6
1st Quarter, 1906	1,264	228	218	140	85	82	40

FILTERED THAMES WATER

(*The same results stated as percentages*).

Time of Year.	10 c.c.			1 c.c.		
	Percentage Positive Results.			Percentage Positive Results.		
	Presumptive.	Confirmatory.	Flaginac.	Presumptive.	Confirmatory.	Flaginac.
January, 1906 ...	17·8	17·2	12·8	7·1	6·6	3·8
February, ,, ...	23·7	22·5	14·3	7·8	7·8	4·3
March, ,, ...	12·9	12·5	6·4	5·3	5·0	1·3
1st Quarter, 1906	18·0	17·2	11·1	6·7	6·4	3·1

Presumptive positive = acid and gas in bile-salt glucose peptone (forty-eight hours at 37° C.).
Confirmatory positive = a glucose-fermenting coli-like microbe isolated.
Flaginac positive = *B. coli* with the characters of 'flaginac' *B. coli* isolated (see p. 225).

These tables show that a positive result for the presumptive test practically coincided with the presence of a glucose-fermenting coli-like microbe (*i.e.*, 18 and 17·2 per cent.), but that in only a little more than half the cases did the presumptive test coincide with the presence, or at least the isolation, of a bacillus having the characters of typical *B. coli*.

The neutral-red method, as shown by the writer and confirmed by Gage and Phelps (Gage and Phelps, 1902, *b*), is a fairly sensitive preliminary test, at least for certain classes of water. As a presumptive test alone and without isolation of the coliform organism, it is unreliable and should not be used. It has, however, quite recently been strongly advocated by Braun (Braun, 1906).

Irons (Irons, 1902), in a careful parallel investigation, compared the results obtained with the dextrose fermentation tube and with neutral-red broth. In 285 determinations from 45 waters, 35 per cent. of positive results were obtained

with the fermentation tube, and 47 per cent. of positive results with the neutral-red method. Irons concluded that, 'although neutral red gives approximately accurate determinations when only *B. coli* is present, the results obtained in the examination of a water for *B. coli* by the neutral-red method alone are likely to be misleading, the tendency obviously being to give too high an estimation of the number of the organism present.'

A consideration of all these so-called 'presumptive tests' forces the conclusion that none of them are—at least, at present—reliable in themselves, either to indicate that a water is good or bad, or that it contains or is free from *B. coli*. These preliminary tests have a great value in that they *indicate* the *probable* numerical presence of *B. coli*, and so save the labour of plating *all* the different dilutions made. This is their object, and as such they are of extreme value, but they are not sufficiently reliable to take the place of the isolation and numerical determination of *B. coli* itself. This is equally true whether the presumptive test is glucose fermentation in a Smith's tube, the neutral-red change,* or the alteration in bile-salt broth. They have only a limited applicability. The American Standards Committee (1905) remarks (p. 91) under presumptive tests: 'Partial tests for *B. coli*, by which several, but not all, of the foregoing characteristics of the organism are ascertained, are considered useful under some circumstances, especially where the time allowed for making the test is necessarily limited. As a general rule, however, where tests for *B. coli* are needed, it is believed that it is unwise to use methods giving less definite results than those described above'—that is, the isolation of the coliform bacillus and a determination of its characters.

* The writer is constrained to emphasize this, since he has been quoted, quite incorrectly, as advocating the use of his neutral-red broth method as a presumptive test, and without isolation of the coliform organisms causing the change.

CHAPTER XVII

The Examination of Water for the Typhoid Bacillus

THE isolation of the typhoid bacillus from polluted water-supplies is a matter of extreme difficulty, a fact exemplified by the very large number of methods which have been advocated for carrying it out. The causes of the difficulty are not far to seek. A comparatively delicate organism, with no very definite morphological or cultural characters, has to be isolated and identified from a large number of other organisms, which for the most part thrive better than itself on all media and at all temperatures. In this it contrasts markedly with the allied *B. coli*, the various chemical activities of which supply reliable means for its isolation and identification. When the typhoid bacillus gains access to a water-supply it is almost invariably accompanied by numerous other organisms, particularly by members of the *B. coli* group, rendering its detection especially difficult. It also rapidly dies out in water, so that its survival in numbers sufficient to make its isolation a practical procedure is often of but short duration.

A difficulty which in practice has probably frequently prevented its isolation from a water-supply which has at one time undoubtedly contained it is the fact that, owing to the long incubation period of typhoid fever, attention is usually not directed to the water as a possible source of infection until several weeks after the specific contamination, when all the typhoid bacilli may have died out.

It is comparatively easy to isolate the typhoid bacillus

when mixed with an uncontaminated water, while when the specific pollution is due to typhoid-infected urine the difficulties are not especially great; but when, as is usually the case, the contamination is by specifically-infected fæces or sewage, the difficulties are very considerable, and tax all the ingenuity of the bacteriologist. For a practical method for the isolation of the typhoid bacillus from water to be successful, it is now fairly generally recognised that the two following principles must be kept in view.

In the first place, since the number of typhoid bacilli will probably be small, it is essential that a large bulk of water be examined.

In the second place, in view of the small number of typhoid bacilli likely to be present, and of the similarity between the colonies of this micro-organism and those of many allied bacteria, it is important to have some means of roughly distinguishing the colonies likely to be those of the typhoid bacillus, and the more perfect the means of differentiation, the lighter the labour and the better the chance of finding the specific organism.

The identification of *B. typhosus* from water naturally divides itself into three stages:

I. Preliminary methods, whereby any typhoid bacilli present are obtained in a quantity of fluid small enough to be directly plated.

II. The isolation of the organism in pure culture.

III. The tests necessary to establish its complete identity.

Stage I.—A variety of different methods have been employed:

(*a*) *Filtration.*—The principle of this method is to pass a definite quantity of the suspected water through a sterile filter (Pasteur-Chamberland or Berkefeld) under pressure. The organisms left behind on the filter are brushed into a definite small quantity of sterile water, which is usually $\frac{1}{100}$ or $\frac{1}{200}$ of the original. Thus, for example, a litre of

the water is frequently filtered, and the organism brushed into 10 or 5 c.c. of water. In this way a true concentration of the organisms in the water has been made, all the bacteria in the original sample being theoretically concentrated one or two hundredfold. The practical details, as well as the fallacies and inconveniences of this method, are considered on p. 255 *et seq.*

This procedure keeps the relative proportion of typhoid bacilli to the other bacteria quite unaltered, unless, as is probable, the less resistant and more delicate typhoid bacilli suffer more than the other bacteria, rendering the final proportion still less satisfactory.

This method in actual practice is much less used than it was formerly; it cannot be recommended.

(*b*) *Chemical Precipitation.*—Here the object in view is to cause by the addition of chemicals a bulky precipitate which entangles any typhoid bacilli present, so that the precipitate contains all, or practically all, the bacteria in the water examined and in a small bulk of material. This can then be used for the inoculation of solid media. Obviously the chemicals used must be such as will not injure the typhoid bacillus.

Vallet (Vallet, 1901) described a method in which quantities of 20 c.c. of the water were placed in each of a series of centrifugal tubes, to each of which were subsequently added 4 drops of a saturated solution of sodium hyposulphite and an equal quantity of saturated solution of lead acetate. The mixture was centrifugalized, and the bulky precipitate dissolved by the addition of more sodium hyposulphite. The clear solution was then plated directly upon a modified Elsner medium, and suspicious colonies worked out.

Schüder (Schüder, 1903) modified Vallet's process. He found that lead acetate had a distinctly prejudicial effect upon the typhoid bacillus, but that the hyposulphite was not harmful, so that the latter must be in excess.

In his method, to 2 litres of the water placed in a tall

cylinder (after rapid filtration if necessary) are added 20 c.c. of 7·75 per cent. sodium hyposulphite solution. After thorough mixing, 20 c.c. of a 10 per cent. solution of lead acetate are added. After twenty to twenty-four hours' standing the liquid is poured off from the precipitate. If a centrifuge is available, they can be separated at once. To the precipitate is added 14 c.c. of a saturated solution of sodium hyposulphite, and the mixture well shaken. From this clear solution plates of suitable material are spread and suspicious colonies subcultivated and investigated.

Ficker (Ficker, 1904) advocated ferrous sulphate as the precipitating agent. Two litres of the water are placed in a narrow glass cylinder, and mixed with 8 c.c. of a 10 per cent. soda solution, and then 7 c.c. of 10 per cent. solution of ferrous sulphate are added and stirred in with a glass rod. The mixture is placed in an ice-chest and allowed to stand for a few hours; if a centrifuge is available, the precipitate can be separated at once. The precipitate is transferred to a sterile tube, and about half its volume of a 25 per cent. solution of neutral potassium tartrate is added. The tube is corked and well shaken, and the precipitate is completely dissolved, more tartrate being added if necessary. From this solution large Petri dishes containing Drigalski-Conradi medium are inoculated.

Ficker claims that by this method there is very little loss of typhoid bacilli, 97 to 98 per cent. of those present being carried down with the precipitate.

Willson (Willson, 1905) advocates the use of alum as a precipitating agent, and claims that the chemical details are simpler. A 10 per cent. solution of alum in distilled water is added, until the alum is in the proportion of 0·5 gramme to the litre of water. The mixture is centrifugalized, the precipitate is mixed with about 1 c.c. of the water and spread over Drigalski-Conradi plates.

Also, quite recently, Müller (Müller, 1905) experimented with the different precipitation methods, and recommended

adding 5 c.c. of liquor ferri oxychlorati (German Pharmacopœia) to 3 litres of the water. The calcium salts present are said by Müller to make the addition of alkali unnecessary, but probably the addition of lime-water will also be required to secure a sufficiently bulky precipitate. The precipitate is allowed to settle for half an hour, when the suspernatant water is decanted and the residue filtered through filter-paper. The precipitate remaining on the filter is then transferred direct to plates containing solid media, and examined in the usual way.

(*c*) *Separation by Means of Antityphoid Serum.*—This has been suggested by several workers as a useful method. Windelbrandt added 1 c.c. of the water to each of a series of broth tubes, incubated at 37° C. for forty-eight hours, and then added antityphoid serum. If agglutination took place, the tubes were centrifugalized, and the deposit used for plating. Schepilewsky (Schepilewsky, 1903) treated larger quantities of water in a very similar way.

Altschüler (Altschüler, 1903) adds 1 per cent. of peptone and 0·5 per cent. of salt to the water, and incubates at 37° C. for twenty-four hours. From the surface about 10 c.c. are withdrawn with a sterile pipette and transferred to a special glass tube, closed below by an indiarubber tube and clip. Blood-serum of an immunized animal is added until the serum present in the mixture is in a dilution of 1 to 50. After about seven hours the precipitate is withdrawn from the bottom and transferred to a smaller tube, in which the process is repeated. Ultimately the deposit is distributed over Drigalski-Conradi plates.

Adami and Chopin (Adami and Chopin, 1904) use a very similar method. The water is collected in Winchester quart bottles, and to each bottle of water is added 20 c.c. of a 1 per cent. glucose broth, the mixture being then incubated at 37° C. for eighteen to twenty-four hours. By means of a sterile pipette, 10 c.c. of the water culture are transferred to each of a number of long, narrow tubes prepared from glass

tubing (7-millimetre bore, and length about 50 centimetres). To each tube is added typhoid serum, of known potency, in varying amounts, final dilutions of 1 : 60, 1 : 100, 1 : 150, and 1 : 200 being, in general, advocated. If typhoid bacilli are present, their agglutination rapidly takes place. The tube which contains the highest dilution of the serum which shows a definite precipitate at the end of five to six hours is recommended for selection for further examination. The sediment is washed with sterile water and allowed to settle several times to remove entangled, but not agglutinated, organisms. A loopful of sediment is now transferred to a broth tube, from which media plates are inoculated, the media of Hiss and of Elsner being recommended.

The methods of Altschüler, and Adami and Chopin, are very similar. How far they will prove of practical service in isolating typhoid bacilli from infected water-supplies is at present quite uncertain ; further experience is necessary.

(*d*) *Enrichment Methods.*—In these methods, instead of concentrating the typhoid bacilli actually present in the water into a small workable bulk of fluid, the object in view is to aid the multiplication of the typhoid bacilli, so that they will become sufficiently numerous to be present in a cubic centimetre or less of the water. The group of methods last considered aim at such multiplication in their preliminary stages.

In the earlier enrichment methods the water was either added to ordinary broth, phenol broth, or phenol broth and acid (*e.g.*, Parietti's method and Hankin's method), or the water was converted into a nutrient medium by the addition of peptone and salt. In all cases incubation was at 37° C., except in Vincent's method (Vincent, 1890), which was at 42° C. The incubated medium was then examined for the typhoid bacillus by plating on simple gelatine, carbol gelatine, or other suitable medium.

The incubation at 37° C. prevented the growth of many water bacteria, while multiplication of any typhoid bacilli

present would take place, so that in mixtures of typhoid bacilli and moderately pure water, such as might, for example, result when urine containing typhoid bacilli gained access to a water-supply, these methods would demonstrate the bacillus fairly readily.

In practice, however, it was found that, whether ordinary broth or phenol broth was employed, multiplication of the members of the colon group of bacilli (almost invariably present under the ordinary conditions of specific water infection) also took place, and to a greater extent than that of the typhoid bacillus itself. So much was this the case that the number of typhoid bacilli was *decreased* relatively to the number of these other organisms, and the likelihood of its isolation was in no way improved.

The writer extensively experimented with such methods seven years ago, and always with the result that the isolation of *B. typhosus* from a pure water, to which it had been added in minute numbers, was comparatively easy, but from a highly polluted water extremely difficult, the difficulty being not at all diminished by the preliminary incubation.

The essential requisite of a good method for the isolation of the typhoid bacillus was lacking—that is, some agent which would enable the typhoid bacillus to multiply, while at the same time inhibiting, or, at least, strongly retarding, the development of organisms of the *B. coli* group. Considerable interest was therefore aroused when Roth (Roth, 1903) announced that *B. typhosus* grew readily on agar and in broth media containing caffeine, while in such media the growth of *B. coli* was completely checked. Roth gave no exact particulars as to the strength of caffeine required for this purpose.

Hoffmann and Ficker (Hoffmann and Ficker, 1904) tested the action of caffeine, confirmed Roth's statement, and worked out a method for the isolation of the typhoid bacillus from fæces and from water. For water investigation their method is as follows: To a flask containing

900 c.c. of the suspected water is added 10 grammes of nutrose in 80 c.c. of sterile water and 5 grammes of caffeine in 20 c.c. of water. The caffeine solution must be freshly prepared for each examination. In making it the solution must not be boiled, or loss of caffeine results, but must be dissolved in warm sterile distilled water (a temperature of 80° C. is sufficient). The caffeine solution must be cooled to between 55° C. and 60° C. before it is added. The liquid is well mixed, and then 10 c.c. of a 0·1 per cent. solution of crystal violet, freshly prepared, is added.

The flask is incubated at 37° C. for twelve to thirteen hours, but not longer. Loopfuls are then withdrawn, and spread over Drigalski-Conradi agar in Petri dishes.

For highly contaminated waters less than 900 c.c. should be examined, the amount taken being diluted with sterile tap water to 900 c.c.

In one experiment they took 1,500 c.c. of river Spree water with 63,000 organisms per c.c., and added about 1,600,000 *B. coli* and about 1,800 typhoid bacilli, and from the mixture isolated the typhoid bacillus.

This method is given in some detail, since it has been successfully used to isolate the typhoid bacillus from a water which caused an outbreak of typhoid fever at Prague (Jaksch and Rau, 1904).

The same investigators (Ficker and Hoffmann, 1904) tested a number of caffeine derivatives, but found that 'none of them acted in the same way, the inhibitory influence on the colon group being shown only by caffeine itself.'

The action claimed by Roth for caffeine is a very important one, and has been the subject of considerable investigation.

Kloumann (Kloumann, 1904) investigated this matter. He used three different strains of *B. coli* and three of the typhoid bacillus, of different ages and from different sources. He found that caffeine, even in weak solutions, restrained the free development of *B. typhosus* as well as of *B. coli*,

the restraining action being, however, somewhat greater for *B. coli* than for the typhoid bacillus. He concluded that there is no strength of caffeine which will allow an increase of the typhoid bacillus while at the same time effectively checking the growth of the colon bacillus. Testing the method on typhoid fæces, as given by Hoffmann and Ficker, he obtained the same results as when direct plating on Drigalski-Conradi medium alone was used.

Rietsch (Rietsch, 1904) tested the action of caffeine upon 22 typhoid and 4 colon bacilli. He found that the highest percentage of caffeine which allowed them to grow varied very greatly. Different races showed very varying sensibility to the action of this alkaloid.

Courmont and Lacomme (Courmont and Lacomme, 1904) inoculated 1 per cent. caffeine broth with 11 races of *B. typhosus* and 9 of *B. coli*. Seven of the typhoid strains grew; the other four and all the *B. coli* did not grow. They found the action of caffeine on *B. typhosus* to be very variable, and indeed concluded that certain typhoid bacilli, especially those subcultivated for a long time in the laboratory, are even more sensitive to the action of caffeine than *B. coli*. They isolated typhoid bacilli from the blood of a typhoid fever patient, which would not grow in caffeine broth, while the bacilli isolated from the urine of the same case grew well.

Subsequent investigations of German workers also show variable results, while in this country experience has been distinctly less favourable.

Willson (Willson, 1905), for instance, using broth containing 0·5 per cent. caffeine, found that the two strains of *B. typhosus* tested did not grow, while both the *B. coli* strains grew, although rather feebly.

Birt (Birt, 1905) tested 4 cultures of *B. coli communis*, a laboratory typhoid culture, 26 typhoid strains isolated from the spleens of fatal cases during the previous year, a paratyphoid bacillus, and a number of dysentery and

other bacilli. A 1 per cent. peptone solution containing 0·5 per cent. of caffeine was used, the incubation temperature being 37° C. The laboratory typhoid flourished well. Of the 26 recently recovered enteric strains, only 7 were alive after five days in the caffeine-peptone solution. None of the colon, dysentery, or alcaligenes bacilli survived. Attempts to separate the typhoid bacillus, by Hoffmann and Ficker's method, from water which had been infected with a recently isolated *B. typhosus* culture were unsuccessful.

These results show that the action of caffeine is not the same for all races of *B. typhosus*, and that although its addition may be very useful, it is not a reliable method in all cases.

Drigalski's Method. — A recent method introduced by Drigalski (Drigalski, 1906) may be appropriately mentioned here. In this method, tinned cans of moderate diameter, with overlapping covers and a capacity of 5 to 10 litres, are sterilized, and then filled with the suspected water. The water is left in the cans, *uncovered*, in diffused daylight, for one to two days at room temperature (18° to 20° C.). Then, with a sterile pipette, one or more c.c. are removed from the surface and spread over large plates of Drigalski-Conradi medium. The more contaminated the water, the smaller the amount to be used for each plate. The plates are incubated and examined in the usual way.

Drigalski considers that the small motile typhoid bacilli are likely to be found on the surface, while the heavier saprophytic and putrefactive organisms tend to sink to the bottom. The small fæcal particles also, which would be the most likely to contain typhoid bacilli, are brought to the surface, owing to the gas production resulting from the putrefactive changes.

(*e*) *Separation by taking Advantage of the Motility of the Typhoid Bacillus.*—Cambier (Cambier, 1901) devised a process for isolating typhoid bacilli from water. A Chamberland (F) filter is placed in alkaline salted broth, and the liquid

on one side is inoculated with the suspected water. The whole is incubated at 37° C. for from fifteen to forty-eight hours. Any typhoid bacilli present are supposed to pass through the filter and into the clear broth on the other side. A little of this latter fluid is removed from time to time, examined microscopically, and the agglutination test employed. Cultural identification tests are then proceeded with, if a positive result has been obtained.

This process is mentioned, because it has been extensively tested and quoted; but, on the whole, the evidence shows that it is quite unreliable.

Hume (Hume, 1903) carried out some experiments which showed that under certain circumstances the superior motility of the typhoid bacillus over most races of *B. coli* may enable that organism to be fairly readily separated. No definite method was evolved.

Stage II.—The isolation of *B. typhosus* in pure culture.

Whatever process may have been employed to obtain the typhoid bacillus in a cubic centimetre or less of fluid, for the isolation of the organism in pure culture solid media must be used. The ordinary laboratory solid media (nutrient gelatine and agar) have been employed, and, in addition, a large number of special media have been advocated from time to time, many of which have marked an advance, and have been distinctly useful until displaced by better, while the advantageous properties of others seem to have been reserved for their originators.

The media which have been employed can, for convenience, be separated into a number of groups.

1. *Plain Nutrient Gelatine and Agar Media.*—When these media are used for the isolation of the typhoid bacillus the characters of its colonies upon gelatine and agar are entirely relied upon, and without intentional retardation of the development of any organisms (apart from temperature influences). These media have the advantage that no in-

hibitory agents are employed which might prejudicially affect any typhoid bacilli present; but they have the great disadvantage that, since no bacteria are suppressed, the plates are very crowded, unless only a fractional quantity of water or concentrated material is employed. When to this is added the fact that the differences between the colonies of *B. typhosus* and the various kinds of *B. coli* are trifling, and frequently not to be made out, it is obvious that the use of such media cannot be other than highly unsatisfactory.

2. *Phenol-Gelatine and Phenol-Agar Media.*—Chantemesse and Widal in 1887 suggested the use of gelatine media containing 0·25 per cent. of carbolic acid. This proportion of carbolic acid was shown by Holz and others to be markedly detrimental to the typhoid bacillus. Parietti used hydrochloric acid as well as carbolic acid.

The addition of smaller quantities of phenol has been used by many workers, particularly by Klein. A percentage of 0·05 has been extensively used. All phenol media for this purpose are most unsatisfactory. The phenol only prevents a number of organisms, few of which are likely to be confounded with the typhoid bacillus, from developing on the plates, while the differentiation of the typhoid bacillus from organisms of the colon group is not in any way aided by its addition. Excess of carbolic acid suppresses the typhoid bacilli; quantities small enough not to injure *B. typhosus* have but a trifling inhibitory action. Most bacteriologists have abandoned its use.

3. *Modified Potato Media.*—The differences between the growth of *B. typhosus* and *B. coli* upon potato were at one time regarded as fundamental, and very important.

Holz advocated the use of a potato gelatine medium containing carbolic acid. This was improved by Elsner (Elsner, 1896), and Elsner's medium was for a number of years used by numerous workers, and was said to be of considerable value. It consists of a potato gelatine containing 1 per cent. of potassium iodide. While both

typhoid and colon bacilli grow well on this medium, the colonies of the two organisms present characters which enable them to be readily separated. Unfortunately, these characteristic differences are not constant, and the method, after extensive trial, has been generally dropped in favour of quicker and more reliable methods.

A potato medium has also been introduced by Weil (Weil, 1901), while Rémy (Rémy, 1901) has made up a gelatine medium with a chemical composition almost identical with that of potato. None of these media can be advocated at the present day.

4. *Specially-combined Media.*—Rosenthal in 1895 studied colony variation in semi-solid gelatine. Klie in 1896 called attention to thread-forming and spreading colonies of the typhoid bacillus in low-percentage gelatine media. Stoddart in 1897 advocated a mixed gelatine and agar medium. Piorkowski in 1899 recommended a urine gelatine medium, with only 3·3 per cent. gelatine. In this medium the typhoid colonies are flagellated.

Hiss (Hiss, 1897 and 1902) experimented with mixed gelatine and agar media, and found them of value in the isolation of *B. typhosus*.

These media are interesting as illustrating one of the directions which research for a suitable medium has taken, but none of them can now be advocated for practical use, except possibly the medium of Hiss, which is still used in America.

5. *Coloured Media, with or without Inhibitory Agents.*—Uffelmann in 1891 appears to have been the first to use a coloured medium for the isolation of *B. typhosus*. He used a gelatine medium containing citric acid and methyl violet, *B. typhosus* and *B. coli* both growing upon this as blue colonies. In practice this method was very unsatisfactory. Recently a number of coloured media have been advocated, most of which are still upon trial, but several have established their claims, and are of proved utility.

Drigalski-Conradi Agar (Drigalski and Conradi, 1902).—This medium was primarily intended for the isolation of the typhoid bacillus from excreta; it is, however, of great value in water bacteriology. It is prepared as follows:

(1) *Agar Preparation.*—To 3 pounds of finely-cut horse-flesh* add 2 litres of water; allow the mixture to stand until next day. Boil the expressed meat-juice for one hour and filter; add 20 grammes peptone sicca (Witte), 20 grammes nutrose, 10 grammes sodium chloride, boil the whole again for one hour and then filter. Now add 70 grammes bar agar, boil for three hours (or one hour in the autoclave), render slightly alkaline (indicator, litmus-paper), filter, boil for half an hour.

(2) *Litmus Solution.*—Litmus solution† (Kubel and Tiemann), 260 c.c.; boil for ten minutes; add 30 grammes of chemically pure milk-sugar and boil for fifteen minutes. Add the hot litmus milk-sugar solution to the liquid agar solution (cooled to 60° C.); shake well; render it again faintly alkaline; then add 4 c.c. of a hot sterile solution of 10 per cent. water-free soda and 20 c.c. of a freshly prepared solution of 0·1 gramme crystal violet (B. Höchst) in 100 c.c. warm sterile distilled water. The result is a meat-water peptone nutrose agar, containing 13 per cent. litmus and 0·01 per 1,000 crystal violet. The medium can be kept in tubes, or in small flasks containing enough for three or four plates. It is sufficient to sterilize once in current steam for thirty minutes.‡

The Petri dishes used should be large (diameter 15 to 20 centimetres), and about 20 to 25 c.c. should be poured

* Beef answers equally well.

† Purified litmus solution made as described in the Appendix, p. 267, is satisfactory.

‡ Eyre (Eyre, 1904, *b*) has carefully investigated the preparation and uniformity of this medium, and has prepared a modified *nutrose agar*, which appears to have several advantages over the original preparation.

into each. The medium should never be less than 2 millimetres thick. After pouring, the plate should remain uncovered for at least one hour, until the steam has evaporated and the agar is quite stiff. A sterile glass rod (Fig. 10, p. 224) bent near one end at right angles is used for smearing the plates. After the plates are spread they should remain open for at least half an hour until the agar surface is completely dry. This, according to the authors, is important, moisture causing the colonies to run together. Saprophytic air organisms are said not to grow, on account of the crystal violet, so that air contamination does not take place.

After fourteen to sixteen hours at 37° C.—twenty-four hours in some cases—the colonies can be distinguished from one another. The coli colonies are red, not transparent, and have a diameter of 2 to 6 millimetres, but considerable variation in size and degree of colour are met with. The *B. typhosus* colonies are blue, with a violet tinge; they are transparent and resemble dewdrops, and have a diameter of 1 to 3 millimetres, seldom larger.

Bile-salt agar and *neutral-red bile-salt agar* (see p. 221) may also be used for the isolation of *B. typhosus*.

Alkaline Litmus Glucose Agar.—Firth and Horrocks (Firth and Horrocks, 1902), in their investigations upon the typhoid bacillus in soil, used a litmus glucose agar of an alkalinity exactly equal to 8 per cent. of decinormal alkali. A 2 per cent. glucose agar was prepared and rendered exactly neutral (Tiemann-Gärtner's neutral litmus being used as the indicator), and then 10 c.c. of the medium were placed in each test-tube. When required for use the tubes were melted in a water-bath, and to each, while still nearly at boiling-point, 1·5 c.c. of neutral litmus solution and 0·92 c.c. of decinormal alkali were carefully added. The medium was poured into plates, which were then incubated for twenty-four hours at 37° C. before use, the object being to ensure that the agar plates were dry, and to see that they were sterile. The *B. coli* colonies were of a bright red colour,

while the colonies of *B. typhosus* were glistening translucent points varying from a flesh-rose to a wine-red colour.

Fuchsin Agar.—Another method advocated for the isolation of the typhoid bacillus is that of Endo (Endo, 1904) of Tokio. This is a fuchsin lactose agar decolourized by sodium sulphite. It is prepared as follows: To 1 litre of water is added 500 grammes of minced beef, 10 grammes of peptone, 5 grammes of sodium chloride, and 30 grammes of agar. The mixture is well boiled, then filtered and neutralized, and made alkaline by the addition of 10 c.c. of a 10 per cent. solution of sodium carbonate; 10 grammes of chemically pure lactose and 5 c.c. of a 10 per cent. fuchsin solution in 96 per cent. alcohol are added. The medium becomes dark red in colour. Twenty-five c.c. of a 10 per cent. sodium sulphite solution are now added. The mixture becomes gradually decolourized, but only becomes quite colourless when the agar solidifies. It is tubed in quantities of 15 c.c., and sterilized once for thirty minutes in current steam.

Certain precautions are described by Endo as necessary. The milk-sugar must be chemically pure, the sodium sulphite either kept in a well-stoppered bottle or be freshly prepared, and the alcoholic fuchsin solution filtered before use. The finished medium must be preserved in the dark, otherwise the light turns it red.

To use, the medium is poured into Petri dishes, solidified, and the material spread with a glass rod in the usual way. The plates are incubated at 37° C. The distinction between the colonies is very marked after twenty-four hours. The coli colonies are bright red, round, and have prominent margins; the typhoid colonies are round, colourless, very transparent, and have thin margins.

A modification of Endo's method has been used by Gaehtgens (Gaehtgens, 1905). He added 0·33 per cent. of chemically pure crystalline caffeine to Endo's fuchsin agar which had an alkalinity equal to 1·5 per cent. normal NaOH

solution *below* the neutral point of phenolphthalein. The caffeine was to inhibit the growth of *B. coli*. Gaehtgens, as the result of a considerable number of experiments, found this addition a great aid in isolating the bacilli from stools. He made 100 examinations of the stools of 60 cases, isolating the bacilli in 37 per cent. by litmus agar, 48 per cent. by fuchsin agar, and in 66 per cent. by caffeine fuchsin agar No experiments on the isolation of the typhoid bacillus with this medium from water are recorded by him.

Still another coloured medium for the isolation of the typhoid bacillus is malachite green agar. This agar was first employed by Loeffler in 1903, who found that *B. coli* did not grow on it, while the typhoid bacillus grew well. It was investigated by Lentz and Tietz (Lentz and Tietz, 1903 and 1905), who advocated for the isolation of the typhoid bacillus from stools a modified malachite green agar, and also Drigalski's medium.

Malachite green agar is prepared as follows by Lentz and Tietz. Three pounds of fat-free ox flesh are finely cut up and macerated with 2 litres of water for sixteen hours. The extract is expressed, boiled for half an hour, filtered, 3 per cent. agar added, and the mixture boiled for three hours. Then are added 1 per cent. peptone, 0·5 per cent. sodium chloride, and 1 per cent. nutrose in 240 c.c. of cold water (the nutrose may be omitted). This mixture is brought to the litmus neutral point by soda solution with duplitest paper, then boiled for one hour and filtered through linen. The reaction of the finished agar is sometimes distinctly acid. It is filtered into small flasks of 100 to 200 c.c., and sterilized three times before use in the usual way. Before the addition of the malachite green, the hot agar is tested by duplitest paper, and made alkaline with sterile soda solution until the red slip is red-violet. To 100 c.c. of the hot agar 1 c.c. of a 1 : 60 solution of malachite green in distilled water (the solution keeps good for ten days) is added—*i.e.*, the agar contains 1 : 6,000.

The finished agar is poured at once into Petri dishes in layers 2 millimetres thick. The dishes are well dried, and can be kept in the ice-chest.

By this strength of malachite green the growth of most kinds of *B. coli*, as well as of many alkali-forming organisms, is greatly diminished. The *B. typhosus* colonies are also retarded, but can be recognised, the size of a grain of sand, with the naked eye after twenty-four hours; after a longer period (two to four days) larger, better developed colonies appear, which colour the agar yellow.

The authors recommend the following procedure: Small portions of the excreta, or concentrated water, are spread by means of a glass spatula over malachite agar and Drigalski-Conradi plates. All the plates are incubated at 37° C. for sixteen to twenty hours. If characteristic colonies are present they are worked out, but if absent from both kinds of plates, about 8 to 10 c.c. of a 0·85 per cent. sterile sodium chloride solution is poured over each malachite green plate, and allowed to stand for about two minutes. The purpose of this procedure is to diffuse the more delicate typhoid and paratyphoid colonies, which are more readily loosened than the thicker and heavier coli colonies. The plate is tilted, and 1 to 3 drops from the surface are transferred to, and spread over, two Drigalski-Conradi plates. These are then incubated and examined for typhoid bacilli. This enrichment method is said to be especially useful for paratyphoid bacilli.

Loeffler (Loeffler, 1906) has recently introduced a 15 per cent. malachite green gelatine and other modifications.

In regard to the respective value of these coloured media, they have all been devised so recently that sufficient evidence is not yet available to discriminate between those which are really of service and those for which the fallacies of identification and the difficulties of manufacture outweigh any differentiating properties. As Greig (Greig, 1906) remarks, in a valuable paper on the

methods employed in Germany in the campaign against typhoid fever (p. 122): 'Each method has its advantages and disadvantages, but it is quite certain that rapidity in detecting the typhoid colonies is largely a matter of experience and practice, and an observer who is accustomed to recognise it on one medium may fail to do so on another.'

The Drigalski-Conradi medium has been subjected to considerable investigation, and on the whole with very favourable results. Klein and Houston (Klein and Houston, 1902-1903) investigated the suitability of this medium for the isolation of the typhoid bacillus from enteric excreta. They found well-marked differences between the typhoid and coli colonies, but point out that not all the blue colonies are those of *B. typhosus*, and that their differentiation must needs occupy some time. Klein (Klein, 1905) subsequently described the different kinds of blue colonies, other than those of the typhoid bacillus, met with by him on this medium.

The writer has used this medium extensively for the isolation of *B. coli*, and also for the identification of typhoid bacilli artificially added to different soils. His experience, which seems also to be that of most workers in this country, is that it is a great labour-saving medium, since the majority of *B. coli* are at once excluded; but that it is impossible to certainly identify the typhoid bacillus on it, and that it is necessary to subcultivate and examine all suspicious colonies, since not all translucent blue colonies are those of the typhoid bacillus.

In regard to the utility of Endo's method, published opinions show considerable variability. The following papers may be consulted: Ruate, 1904; Petkowitsch, 1904; and Marschall, 1905.

Klinger (Klinger, 1906), in a valuable paper, compared the more recent methods for the detection of the typhoid bacillus in stools. He carried out comparative investigations upon 43 stools using for each the four following

methods: Drigalski-Conradi, fuchsin agar (Endo), malachite green enrichment method, and initial cultivation in caffeine broth. Positive results were obtained in 30 cases, the bacillus being isolated on Drigalski-Conradi plates 13 times, on fuchsin agar 16 times, by the caffeine method 20 times, and by the malachite green method 26 times. In only 4 cases were the bacilli found by one or the other procedure, and absent by the malachite green method.

As regards typhoid stools, therefore, Klinger obtained the best results with the malachite green enrichment method, but it is not safe to assume that the procedure which is most suitable for excreta is also most suitable for specifically infected water.

Stage III. The tests necessary to establish the identity of the isolated bacillus.

It is now generally advocated that all suspicious organisms should first be roughly tested as to their ability to be agglutinated by typhoid serum in moderate dilution. If no agglutination takes place, they may be at once rejected. If a positive reaction is obtained, cultural tests are carried out, while a more exact series of agglutination tests is made. In this way it is possible to greatly lighten the labour of examining a large number of suspicious colonies. The colonies under investigation are either at once examined or after subcultivation. For the first method a little of the colony is transferred to a cover-slip emulsified in a drop of broth, and an equal quantity of serum added, the whole test being carried out on the cover-slip. For the second method the colonies are inoculated into nutrient broth, and the agglutination tests performed next day, after growth has taken place. The latter is, on the whole, preferable. The broth cultures which show a positive result are then ready for further investigation; the others are rejected.

The tests necessary to establish the identity of an organism as *B. typhosus* have been already considered (see p. 94).

With all the bewildering methods of isolation which have been advanced, the inexperienced worker may have some difficulty in selecting a suitable method should he be confronted with the necessity of looking for the typhoid bacillus in water. As already pointed out, experience in the appearance of the colonies and in the steps of the different methods is essential to success, and it is better to know one method well than to experiment with several, with only a limited acquaintance with each.

Great as are the improvements which have taken place in the facility with which typhoid bacilli can be isolated from specifically infected excreta, with none of the different methods can it be said that the isolation of the bacillus from an infected water-supply is other than a difficult and unsatisfactory procedure, and only under very favourable conditions can success be hoped for.

It is advisable to try several methods, and in the writer's opinion the following would probably be the most serviceable procedure:

(*a*) Examine 5 to 10 litres by Drigalski's method.

(*b*) Take 1 litre of the water and precipitate the organisms by Ficker's, or one of the other precipitation methods. The precipitated organisms are distributed over a large series of Drigalski-Conradi plates. Fuchsin agar and malachite green agar plates (enrichment method) might also be employed if facility had been acquired in their use.

(*c*) Examine another litre or more by Hoffmann and Ficker's caffeine method, the precise directions being followed.

All suspicious colonies obtained from the three methods (and the total number may be large) are subcultivated into broth, and incubated at 37° C. until next day. They are all then examined in hanging drop, and those which show actively motile bacilli are tested with antityphoid serum. A fairly powerful serum should be available, and a dilution of not less than 1 per cent. should be employed.

All those which fail to show agglutination are rejected, while those reacting are each subcultivated into litmus-milk, glucose neutral-red broth (in a Durham tube), and lactose-peptone solution (in a Durham tube).

All the organisms giving cultural characters in these media which accord with those of *B. typhosus* (see Chapter VII.) are fully worked out. The tests should include accurate and extended agglutination tests with highly dilute sera. Such organisms will usually be found to be very few in number.

Some such procedure as the above will rapidly decide whether any typhoid bacilli have been isolated. Usually these few tests will have promptly rejected every suspected organism as not being a typhoid bacillus.

CHAPTER XVIII

The Examination of Water for other Intestinal Organisms

Examination for B. Enteritidis Sporogenes.

Even in waters from contaminated sources this organism may be present in only small numbers, so that it is usually necessary to examine a considerable volume of the water. Convenient amounts to examine are 10, 100, 500, and 1,000 c.c. In order to deal with these large volumes, some method by which the micro-organisms in the water can be concentrated has to be employed. This is invariably done by filtering the water through a porcelain filter, the water passing through and leaving the contained bacteria deposited upon the filter. They are then brushed off, emulsified in a small quantity of water, and definite quantities of this are used for the determination of the presence of this organism.

Filters for this purpose are either of the Pasteur-Chamberland variety, made of unglazed porous 'biscuit' porcelain, or Berkefeld filters made of kieselguhr.

After use, particularly if used for the filtration of liquids containing a considerable amount of organic matter, such as highly contaminated waters, these candles require, in addition to ordinary sterilization, to have the organic matter burnt out by ignition, otherwise the pores become clogged and filtration is very slow. This is best carried out by heating in a muffle furnace, the candles after ignition being

cooled very gradually to prevent fracture. In the absence of a furnace, a makeshift is to suspend the candle by wire from a retort stand and heat with two or three large Bunsen burners for some hours. This is fairly effective.

Two kinds of candles may be employed. In one, filtration is from within out, in the other from without in.

In all cases, in order to retain the bacteria on the filter, the pores of the candle must be very minute, so that filtration is very slow. To accelerate it, aspiration or pressure, or both, may be employed.

Combined pressure and aspiration filtering apparatus has been designed and used by Pakes, and also by Houston. They insure more rapid filtration, but are costly, and not essential for ordinary work; they are not recommended.

Of the two procedures, filtration from within out is preferable to filtration from without in, as in the former method enough water can be left in the candle, or readily added, so that the emulsion is made with greater ease and less danger of outside contamination than when the organisms have to be brushed off from the outside into a definite quantity of sterile water.

The two forms of apparatus described are convenient.

The simple arrangement (Fig. 12) of A. W. and M. W. Blyth* is very useful. It is described by them as follows: 'A is a piece of stout glass tubing about 2 feet long. The Berkefeld filter-candle B is fitted into this by means of the tightly-fitting rubber ring C. The nozzle of the candle is directly connected by means of a short piece of pressure tubing with a glass tube passing into the pressure flask E, which is in its turn connected with a filter-pump. A litre flask of the water to be examined is inverted into the tube A, round the top of which sterile cotton-wool is packed. As the level of the water in the tube falls, bubbles of air pass up into the flask, and so keep the

* A. Wynter Blyth and M. W. Blyth, 'Foods: their Composition and Analysis,' 1903, p. 563.

level of the water in the tube constant until nearly all the water is filtered. The inverted flask and cotton-wool prevent the water from becoming contaminated with air organisms during filtration. All the parts of this apparatus are easily sterilized.

'After filtering the water through the sterilized apparatus,

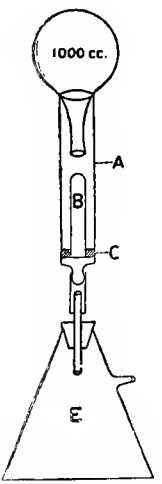

FIG. 12.

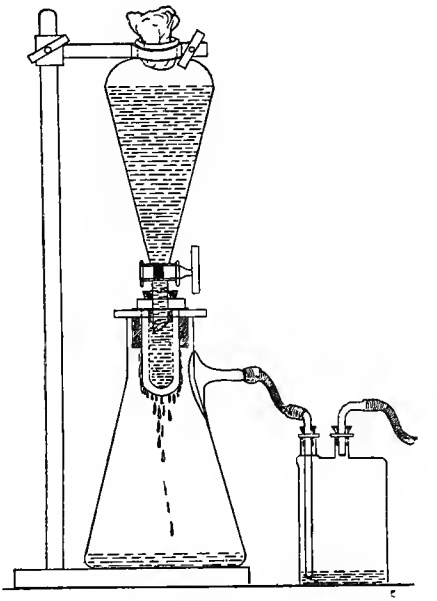

FIG. 13.
From Eyre's ' Bacteriological Technique.'

the candle is taken out, and 10 c.c. of sterile water are measured into a sterile glass dish; the whole surface of the candle is well brushed into these 10 c.c. of water by means of a thoroughly clean sterile nail-brush.'

Another form of apparatus which is also convenient is figured (Fig. 13). In this apparatus the water is placed in a separation funnel which is connected with the filter-candle by means of a perforated rubber stopper. The filter-flask

is connected to an exhaust pump. In this arrangement the filtration is from within out, so that all the bacteria are deposited upon the inside of the candle. After filtration sterile water is added and an emulsion made. The porcelain filter-tube should hold rather over 10 c.c. of water.

Both forms of apparatus must be sterilized in steam for several hours before use.

The time taken to filter a litre varies considerably, the unequal amounts of organic matter in the water being the chief cause of variation; if abundant, filtration may be very slow.

The two forms of apparatus described illustrate simple and convenient arrangements; other methods of connection will readily suggest themselves.

Whatever arrangement is adopted, in all cases the deposited bacteria are made into an emulsion with a definite quantity of sterile water, 5 or, more usually, 10 c.c. of water being employed. Varying amounts of the emulsion are added to the tubes of media required for the different examinations. If the bacteria in 1 litre are concentrated into 10 c.c., 1 c.c. of the emulsion is considered to contain the organisms of 100 c.c., 0·1 c.c. of 10 c.c., etc.

In the examination for *B. enteritidis sporogenes*, convenient amounts of the emulsion to examine are 0·1, 1·0, 5 c.c., and the remainder (about 4 c.c.) representing respectively 10, 100, 500, and about 400 c.c. of the original water. For the actual examination milk-tubes are used. These should contain at least 10 c.c. of sterilized whole-milk, and must be boiled for half an hour before use to expel the contained air; they are then rapidly cooled.

Definite quantities of the original water, or emulsion, are then added by means of a sterile pipette, and well mixed. The inoculated milk-tubes are heated to 80° C. in a water-bath, and kept at that temperature for ten minutes, to kill all non-sporing forms. The ordinary water-bath can be easily regulated to remain at 80° C. for this purpose.

The tubes are then removed, incubated anaërobically at 37° C., and examined after two days for the characteristic milk changes. These changes are described on p. 116.

For anaërobic incubation wide-mouthed bottles with solid indiarubber stoppers are very convenient. They can be made to take half a dozen tubes. Pyrogallic acid in powder is placed at the bottom, the inoculated milk-tubes are inserted, and a quantity of a strong (40 per cent.) solution of KOH is poured in through a funnel just before the stopper is inserted.

The whey of the milk-tubes showing the characteristic 'enteritidis change' should be examined microscopically. It should contain large bacilli which do not contain spores.

If pathogenicity is to be tested, 1 c.c. of the whey is injected subcutaneously into a guinea-pig.

In this way the numerical distribution of this organism in the sample is ascertained.

A number of observers have pointed out that it is not necessary to incubate the milk-tubes anaërobically to obtain the typical change. That special anaërobic precautions can be omitted is no doubt due to the fact that the thick layer of cream on the surface of the milk insures anaërobiosis. If separated milk is used without providing an oxygen-free atmosphere, the characteristic changes do not take place. Many workers, therefore, use whole-milk, but do not otherwise provide special anaërobic conditions. Hamilton suggests the use of a layer of olive oil over the medium, and Thresh pours melted vaseline over the surface of the milk to a depth of about $\frac{1}{2}$ inch before heating to 80° C., and uses no further precautions to prevent air access.

Hewlett (Hewlett, 1904) has recently pointed out the inconvenience and fallacies of filtration methods, and suggests the following method: 'Boiling tubes, measuring about 17 centimetres total depth by about 3 millimetres in diameter, have 40 c.c. of milk introduced into each, and are

plugged with wool and sterilized in the ordinary way. At the time of using, the tubes are boiled in a water-bath for a few minutes to expel air, and then 60 c.c. of the water to be examined are added to each—that is, the milk is diluted with one and a half times its own volume of water. The wool plug is replaced by a cover of two thicknesses of sterile filter-paper, kept in place by a rubber band, and the tubes are heated to and maintained at 80° C. for ten to fifteen minutes. The tubes are then incubated anaërobically at 37° C. in a Bulloch's apparatus, or, what answers just as well, with alkaline pyrogallic in a stoppered museum jar without a neck, of a size adapted to contain them. By using a dozen tubes, 700 c.c. of water can be examined. Tubes have been used in preference to flasks, as the former are more readily contained in the jars or apparatus employed for the anaërobic cultivation. Other modifications will readily suggest themselves.'

Examination for Streptococci.

The detection of the presence of streptococci and their numerical estimation in water is, at the present day, only carried out by a few bacteriologists.

Houston, to whom is due the credit of first pointing out the value of the detection of these organisms in water, used—at least, for most of his work—the following method (Houston, 1900-1901, *b*):

A litre of water is passed through a sterilized Pasteur filter. The organisms from the surface of the filter are brushed with a sterile brush into 10 c.c. of sterile water. Surface agar plate cultures are made by spreading 0·1 c.c. of the emulsion (equal to 10 c.c. of the original water) over an agar plate. In the case of contaminated waters, plates are spread direct with the original water. The plates are examined after twenty-four hours' incubation at 37° C. The minute colonies present are examined under a low

power of the microscope, and each is subcultivated into a broth tube. These are microscopically examined after incubation at 37° C. Those showing streptococci are further investigated.

The chief tests employed by Houston were, morphology, characters of surface gelatine plate colonies, surface agar plate colonies, growth in broth and in litmus-milk, appearance on gelatine slope, and the result of inoculation into mice.

Houston has recently pointed out that the medium of Drigalski and Conradi is well adapted for isolating streptococci, the minute colonies being subcultivated into broth in the same way as from ordinary agar.

Lactose litmus agar is also a suitable medium for their isolation. They grow on this as small circular red colonies.

In the writer's opinion, the best method of determining the numerical distribution of streptococci in a water is to add varying quantities of the sample—*e.g.*, 0·1, 1·0, and 10 c.c.—to tubes of glucose broth, and to add quadruple strength glucose broth to a larger quantity—*e.g.*, 40 or 100 c.c., according to the amount collected.

The writer, in actual practice, adds 0·1 and 1·0 c.c. of the water to tubes of glucose neutral-red broth, 10 c.c. to a tube of double strength neutral-red broth, while to the 40 c.c. or so left in the 2-ounce sample bottle 10 c.c. of four times strength glucose neutral-red broth are added.

Neutral-red broth is used, since the streptococci are more readily visible in it, while, since this medium is used by the writer for other parts of the examination, it is also more convenient than keeping a separate stock of glucose broth.

The tubes are incubated at 37° C. for forty-eight hours, and are then examined microscopically in hanging-drop preparations.

The broth tube containing the quantity of water next below the one giving a positive result is examined several times in fresh preparations. In the rare cases in which it is doubtful whether streptococci in chains are actually

present, a little of the fluid is centrifugalized and the deposit stained by methylene blue.

In this way the numerical distribution of streptococci as such, and without regard to their biological characters, can be readily ascertained. Only cocci which occur in definite chains should be regarded as streptococci.

At the present day, with the limited knowledge available, the value of the streptococcus test for excretal contamination does not rest upon the presence of any one variety of streptococcus, but upon *streptococci as a class*. As regards the sanitary opinion upon a particular sample, the isolation and determination of the characters of the streptococci present adds no information which can influence the opinion to be derived from their presence. From this point of view, therefore, it is unnecessary to isolate, but from the point of view of research it is very desirable that all streptococci should be isolated and their characters recorded, since the value of the streptococcus determination must ultimately rest upon the kinds of streptococci present, and not, as now, upon the mere presence of members of this group of organisms.

The streptococci are best isolated from the glucose-broth tubes by adding a platinum loopful of the broth to a lactose litmus agar plate, and distributing it over the surface. All suspicious colonies are subcultivated and worked out.

A conceivable drawback to this method of isolation is that the preliminary broth incubation may lead to the multiplication of the less significant strains of streptococci and a suppression of the more significant.

At the present time it is not possible to say what the characters are which it is most important to investigate. For research purposes, the more extensive the tests the greater the value of the observation. These tests are discussed in Chapter VIII.

NOTE.—Streptococci that have been isolated frequently die out rapidly in or on the ordinary media, and so cannot be kept for com-

parison with streptococci from other sources. The writer has found that—at least, for all the strains tested by him—they can be kept alive for very long periods in ordinary glucose broth containing excess of calcium carbonate. Such a medium was suggested by Hiss for obtaining streptococci, pneumococci, etc., in large bulk. It differs from ordinary glucose broth only in the fact that each tube contains a little solid calcium carbonate. These chalk-broth tubes are sterilized in the ordinary way. After inoculation and two days' incubation at 37° C., they are kept at room temperature. Streptococci have been kept alive in this way for over six months without difficulty.

Examination of Water for Spirillum Choleræ.

Of the different methods suggested, the simplest and most satisfactory is the enrichment method employed by Koch and others. This is best done by converting the water into a nutrient medium by the addition of peptone and salt.

About a litre of the water is placed in twelve large sterile Erlenmeyer flasks, 90 c.c. in each. To each is added 10 c.c. of a sterile solution, consisting of 10 per cent. peptone and 5 per cent. sodium chloride.

The flasks are then incubated at 37° C. After eighteen hours' incubation, microscopic preparations and examinations in hanging drop are made from the surface of each flask. The medium is one in which the cholera spirillum grows very rapidly, and if present, it shows itself in the very thin pellicle on the surface of the liquid, often before the other organisms have had time to develop. The flasks which show the presence of vibrios are used to inoculate agar and gelatine plates, a loopful of the fluid being withdrawn from the surface for this purpose. Suspicious colonies on the agar and gelatine plates are subcultivated upon agar slope, and their characters studied in pure culture.

A large number of spirilla very similar to the cholera organism have been found in water, and all available tests must be employed, including, in particular, Pfeiffer's test. For particulars of these tests see p. 119 *et seq.*

Only vibrios which possess the cultural characters of the cholera spirillum, and which react positively to Pfeiffer's and the agglutination tests, can be classed as true cholera organisms.

True cholera vibrios have been isolated in many instances from infected water-supplies.

APPENDIX

Summary of Procedure recommended for the Bacteriological Examination of a Sample of Water

THE following is liable to modification according to the water-supply examined, but is suitable for most cases.

Collection.—Not less than 2 ounces to be collected in a sterile glass-stoppered bottle.

For precautions to take during collection, see p. 198.

Examine within three hours, or transmit packed in ice.

Inoculations.—Everything must be in readiness before the examination is started—*i.e.*, gelatine and agar tubes melted and at a suitable temperature, other media tubes ready, sterile pipettes, and Petri dishes at hand. Use a 1 c.c. pipette graduated in $\frac{1}{10}$ c.c. Mix the sample thoroughly.

Add 0·2, 0·3, and 0·5 c.c. respectively to three gelatine tubes; roughly label with a paraffin pencil.

Add 0·1 and 1·0 c.c. respectively to two agar tubes; roughly label, and replace at once in the hot-water bath.

Add 1·0 c.c. to a tube of bile-salt broth.

Add 10·0 c.c. to a tube of double strength bile-salt broth.

Add 0·1 c.c. to a tube of glucose neutral-red broth (in Durham's tube).

Add 1·0 c.c. to a tube of glucose neutral-red broth (in Durham's tube).

Add 10·0 c.c. to a tube of glucose neutral-red broth double strength.

To the water remaining in the bottle add the contents (about 10 c.c.) of a tube of four times strength neutral-red broth. Replace the glass stopper.

The tubes as inoculated can be placed in sequence in a test-tube stand by the assistant, so that it is unnecessary to label until all the additions are completed. All the inoculations can be done with the one pipette, and very rapidly.

If, as is frequently useful, a second 2-ounce sample is collected, the second 10-c.c. inoculation should be made from this sample, and subsequently 10 c.c. of four times strength neutral-red broth added to the water remaining in the bottle.

The gelatine and agar tubes are now poured into Petri dishes, after thorough admixture of water and medium has been made.

The Petri dishes are labelled and their contents rapidly solidified—*e.g.*, on a plate-cooling apparatus.

The agar plates are incubated at 37° C. upside down, and the gelatine plates at 20 to 22° C., but not reversed.

The bile-salt and neutral-red broth tubes are labelled and incubated at 37° C.

Examination of Plates and Tubes.

Plates.—For time and method of counting, see p. 205, following the recommendations of the English Water Standardization Committee.

Tubes.—Examine after twenty-four and after forty-eight hours.

For B. coli.—If the 1·0 and 10 c.c. bile-salt and neutral-red broth tubes show no gas or reaction after forty-eight hours, it can be assumed that *B. coli* is absent in these amounts. Then, *in every case*, plate out from the broth and water in the sample bottle.

If gas is present in these smaller amounts, use the one showing gas in the tube with the least quantity of added water for inoculating plates of solid media.

For method of inoculating plates, see p. 223.

For media to use for isolation, see p. 219.

Subcultivate at least three colonies.

Inoculate the following media from these: Gelatine slope, litmus-milk, lactose peptone solution, peptone-water, and glucose neutral-red broth.

For results to record, see p. 226.

For Streptococci.—Examine after forty to forty-eight hours' incubation all the neutral-red broth tubes and the mixture in the sample bottle, in hanging-drop preparation, for the presence of definite streptococci. Isolate if considered necessary (see p. 262).

Note.—The examination for *B. enteritidis sporogenes* is not recommended as a routine procedure.

Recording of Results and Opinion.

The agar and gelatine enumerations, the numerical distribution of *B. coli* ('excretal' and atypical) and of streptococci should all be recorded.

The following example will serve as a practical illustration:

Sample received June 9, 1906. Examined two hours after collection. Not ice-packed. Reported June 20, 1906.

Number of organisms developing at 37° C. = 32 per c.c.

Number of organisms developing at 21° C. = 450 per c.c.

'Excretal' *B. coli* present in 10 c.c., but not in smaller quantities of the sample.

'Excretal' *B. coli* (neutral red and indol −) present in 1 c.c.

Streptococci present in 40 c.c., not found in 10 c.c. or less.

Opinion.—This will vary according to the kind of water. For guiding principles and standards, see Chapter XII.

The reaction and composition of the agar and gelatine, the conditions of count, etc., should be recorded as a private entry.

Note on Reaction Indicators

The chief indicators of use to the bacteriologist are litmus, methyl orange, and phenolphthalein.

In bacteriology, and particularly in water bacteriology, increased attention is being paid to the chemical products of bacteria and an exact determination of their presence and amount. For the estimation of chemical reaction a number of indicators are available, but no one of them is suitable for all cases.

Litmus.—A simple solution may be made by digesting the powdered cubes repeatedly with hot water, mixing the extracts, and, after allowing them to stand all night, decanting the solution from the inert sediment into a clean bottle.

In litmus solution so made, however, a red dye is also present, while calcium and other salts are dissolved out. For bacteriological purposes a pure solution of the blue dye should be used. This is called 'azolitmin.' It is freely soluble in water, but insoluble in alcohol.

It can be conveniently prepared as follows: Weigh out 2 ounces of powdered litmus; digest repeatedly with fresh quantities of hot water until all the colouring matter is dis-

solved out; allow to settle, and decant off the fluid from the insoluble powder. Add together the extracts, which should measure about a litre. Evaporate down the solution to a moderate bulk, then add a slight excess of acetic acid, so as to convert all carbonates present into acetates. Continue the evaporation, the later stages being over a water-bath, until the solution becomes pasty. Add 200 c.c. of methylated spirit, and mix thoroughly. The spirit precipitates the blue colouring matter, while a red colouring matter, together with the alkaline acetate present, remains in solution. Transfer to a filter. Wash out the dish with methylated spirit, and add this to the filter. Wash the precipitate on the filter with methylated spirit. Dissolve the pure colouring matter remaining on the filter in warm distilled water and dilute to 500 c.c. Azolitmin solution prepared in this way is more sensitive than ordinary litmus solution.

A drawback to the use of litmus as an indicator is that free carbonic acid interferes with its action, so that, for example, in titrating acid solutions with an alkaline carbonate, the liberated CO_2 changes the blue to purple before complete neutralization is reached. To avoid this, the liquid must be boiled after each addition to get rid of the CO_2.

Methyl orange may be used as a 0·1 per cent. solution in distilled water. Its chief advantage is that, unlike litmus and phenolphthalein, it is comparatively unaffected by carbonic acid. It cannot be used for the organic acids, such as acetic, oxalic, citric, and tartaric, since the end reaction is indefinite. Nitrites and nitrous acid decompose it, so that it is also inadmissible if such substances are present.

As Sutton * points out, methyl orange is especially useful for the accurate standardizing of any of the mineral acids by means of pure sodium carbonate in the cold, the liberated carbonic acid having practically no effect. It is also excellent with ammonia or its salts. A single drop of the solution (0·1 per cent.) is sufficient for 100 c.c. or more of any colourless solution, the colour being faint yellow if alkaline and pink if acid. If too much is used, the end reaction is slower and much less definite.

All titrations should be carried out at ordinary temperatures if the utmost accuracy is desired.

* F. Sutton, 1904, 'Volumetric Analysis,' 9th edition, p. 34.

Phenolphthalein.—A $\frac{1}{2}$ per cent. solution in 50 per cent. alcohol is almost always used. This forms a colourless solution. In neutral or acid solutions it remains colourless, but becomes purple red with the faintest excess of alkali. It is very useful for titrating all the organic acids. It cannot be used in the presence of carbonic acid or of ammonium salts. If used in fluids from which CO_2 is evolved, the titration must be done at nearly boiling temperature and after the liquid has been boiled.

In titrating such complex substances as nutrient broth, or mixtures containing it, different determinations of acidity or alkalinity are obtained according to the indicator used—that is, some of the constituents which react alkaline or acid to one indicator are not affected by another, and so react neutral.

The following table, from a paper by Eyre (Eyre, 1900), shows the behaviour of some of the phosphates (always present in nutrient broth) to some of the chief indicators:

	Phenol-phthalein.	Rosolic Acid.	Litmus.	Lacmoid.	Methyl Orange.
Phosphoric acid, H_3PO_4	+	+	+	+	+
Monobasic sodium phosphate, NaH_2PO_4	+	+	+	o	o
Dibasic ,, ,, Na_2HPO_4	o	–	–	–	–
Tribasic ,, ,, Na_3PO_4	–	–		–	–

+ = acid. o = neutral. – = alkaline.

From this table it will be seen that when neutralizing media with caustic soda solution, and using litmus as the indicator, the neutral point is reached as soon as the phosphates present have been converted into the dibasic form. The dibasic phosphates react alkaline to litmus, so that their presence will prevent the addition of alkali to complete neutralization. On the other hand, they are neutral to phenolphthalein, so that if this indicator be used additional alkali will be added until complete replacement of all hydrogen atoms is effected.

For media it is found that the neutral point as given by litmus is too acid, while that obtained by using phenolphthalein is too alkaline; hence the adoption of some standard reaction between the two.

The difference in the actions of these indicators depends upon their composition. A. H. Allen has shown (quoted from Sutton, p. 39) that the acid which enters into the composition of an indicator must be weaker than the acid which it is required to estimate by its means. The acid of which methyl orange is a salt is a tolerably strong one, since it is only completely displaced by the mineral acids. The organic acids are not strong enough to overpower it completely; hence the uncertainty of the end reaction. The still weaker acids, such as carbonic, hydrocyanic, boric, oleic, etc., do not decompose the indicator at all, so that their salts may be titrated by it, just as if the bases only were present. On the other hand, the acid of phenolphthalein is extremely weak; hence its salts are easily decomposed by the organic and carbonic acids.

Preparation of Standard Solutions of Acid and Alkali.

Standard solutions are necessary to the bacteriologist for preparing media of standard reaction, for estimating the quantity of acid or alkali produced by the growth of different organisms, and for other purposes. Details of their preparation are included here for convenience of reference.

The standard solutions generally employed for bacteriological purposes are *normal* ($\frac{N}{0}$) and *decinormal* ($\frac{N}{10}$) solutions of KOH and H_2SO_4.

A *normal* solution is made up so that 1 litre of it at 16° C. contains the hydrogen equivalent in grammes of the active reagent.

To prepare these solutions it is advisable to first make a *normal* solution of Na_2CO_3, since this can be done with great accuracy, and then to use this solution to standardize the others.

Preparation of Normal Sodium Carbonate.

Pure sodium carbonate is most readily prepared by heating sodium bicarbonate.

Accurately weigh a platinum basin; weigh in roughly about 47 grammes of pure powdered sodium bicarbonate; heat to dull redness (270° to 300° C.) for thirty minutes, taking care not to fuse the salt; cool in a desiccator, and weigh. This weight, less that of the platinum dish, gives

the weight of pure sodium carbonate. The carbonate is then transferred to a beaker, all traces of Na_2CO_3 in the platinum dish being transferred to the beaker by repeated rinsing with distilled water. The Na_2CO_3 is completely dissolved, then carefully poured into a 500-c.c. flask, and the beaker rinsed with three or four successive changes of water, which are added to the flask. Distilled water is added to bring up exactly to the 500 c.c. mark, the liquid being at 16° C.

Rather more carbonate than would exactly make up 500 c.c. has been taken, so that extra water will have to be added to make up an accurate $\frac{N}{0}$ solution. This amount is readily calculated from the weight of Na_2CO_3 used, and is carefully added from a burette or pipette.

$\frac{N}{0}$ Na_2CO_3 contains 53 grammes per litre, or 26·5 grammes per 500 c.c.

Suppose the weight of Na_2CO_3 used was 27 grammes.

26·5 grammes require to be dissolved in 500 c.c.;

$$27 \quad ,, \quad ,, \quad ,, \quad ,, \quad \frac{27 \times 500}{26{\cdot}5} \text{ c.c.}$$

$$= 509{\cdot}4 \text{ c.c.}$$

That is, 9·4 c.c. has to be added to the 500 c.c. in the flask. The solution is then very thoroughly mixed and transferred to a glass-stoppered bottle, the stopper of which has been greased to prevent its sticking.

This solution must be made up with extreme care, as the other solutions are standardized from it.

Preparation of Normal Sulphuric Acid.

This solution is a semi-molecular one, and contains 49 grammes of sulphuric acid per litre.

Measure out 30 c.c. of pure H_2SO_4, and run it into about 200 c.c. of distilled water in a beaker, stirring constantly; cool; make up to 1 litre with distilled water, and mix thoroughly. The solution is then standardized from the $\frac{N}{0}$ Na_2CO_3, methyl orange being the most convenient indicator.

This can be readily done as follows: Accurately pipette exactly 20 c.c. of $\frac{N}{0}$ Na_2CO_3 into a beaker containing distilled water. The beaker should be placed on a white tile or other white surface. Add one drop of methyl orange solution (0·1 per cent.). Fill a burette, graduated in $\frac{1}{10}$ c.c., with

the unstandardized acid, and run the acid carefully into the beaker until the neutral point, as shown by the indicator, is reached. Repeat with a fresh 20 c.c. of sodium carbonate solution. The results should not differ by more than 0·1 c.c.

From the amount of acid required to neutralize 20 c.c. of $\frac{N}{0}$ Na_2CO_3 the strength of the acid is obtained, and an adjustment to an accurate $\frac{N}{0}$ strength is readily made. As above prepared the solution is too strong.

For example, the 20 c.c. of alkali might require 18·8 c.c. of the acid.

20 c.c. $\frac{N}{0}$ alkali require 18·8 c.c. of the acid;

$\therefore$ 1,000 ,, ,, ,, would require $\frac{18{\cdot}8 \times 1{,}000}{20}$ of the acid = 940 c.c.

That is, 940 c.c. of the acid solution is equivalent to a litre of $\frac{N}{0}$ alkali.

940 c.c. of the acid are accurately measured into a litre flask and diluted with distilled water up to the litre mark (at 16° C.).

The solution is well mixed, and is now of accurate $\frac{N}{0}$ strength.

Standard solutions of sulphuric acid will keep of constant strength for long periods, and can be used for the standardization of all other acids and alkalies.

Preparation of Normal Potassium Hydrate Solution.

This solution contains 56 grammes of caustic potash per litre. Pure KOH (about 29 grammes for 500 c.c.) is dissolved in distilled water. It is then diluted with distilled water and standardized against the $\frac{N}{0}$ H_2SO_4, methyl orange being used as the indicator, and the sulphuric acid being run in from a burette into a definite amount of the diluted alkali solution.

From the amount of $\frac{N}{0}$ H_2SO_4 used the strength of the alkali is determined. It is then diluted with well-boiled, cooled, distilled water to an exact strength of 56 grammes per litre.

If phenolphthalein is used as an indicator, the titration must be conducted after boiling the alkali in the beaker, and at nearly boiling-point.

The solution must be kept well stoppered and exposed to the air as little as possible, as it absorbs CO_2 and loses strength. The glass stopper must be greased, or it will stick.

The solution requires to be restandardized from time to time against $\frac{N}{0}$ H_2SO_4, as it does not keep unchanged so well as that acid.

Decinormal ($\frac{N}{10}$) solutions should not be kept for long periods in bulk, but should be made up fresh from *normal* solutions by accurately diluting 100 c.c. of the latter to a litre with boiled distilled water.

Preparation of Nutrient Media.

The preparation of the more specialized media used for the isolation of certain organisms is given under the appropriate chapters. Methods for the preparation of a number of the more generally used nutrient media will be now described.

Reaction Standardization of Media.

In the first part of this work the importance of working with media of standard reaction was pointed out, and in the note on reaction indicators it was explained that litmus was much less suitable for this purpose than phenolphthalein.

The signs + and − are now very generally used for expressing reactions, and the results are expressed in terms of *normal* acid or alkali per cent. Thus, a +1·0 medium indicates that the medium is acid to the extent of 1·0 c.c. of *normal* acid per 100 c.c. of the medium.

The reaction of +1·0 (equivalent to + 10 Eyre's scale) was adopted by the English Committee on Standard Methods, and is taken as the standard reaction for all media, the addition of alkali being stopped at the first appearance of a pink colour.

The actual standardization is conveniently done as follows:

Ten c.c. of the medium (gelatine, agar, broth) are pipetted into an evaporating basin or small beaker containing about 50 c.c. of hot distilled water. Half a c.c. of phenolphthalein solution is added, and the mixture is boiled for several minutes.

$\frac{N}{10}$ sodium hydrate solution is cautiously run into the

beaker from a burette until the first tinge of pink permanently remains. The amount of alkali added is then read off.

This is repeated with a fresh 10 c.c. of the medium.

Results not differing by more than 0·1 c.c. should be obtained. The mean is taken, and from this the amount of $\frac{N}{0}$ alkali required to neutralize the whole litre of the medium is calculated.

The neutral point, as given by phenolphthalein, is too alkaline; a + 1 per cent. reaction is required. From the calculated amount of normal alkali required, 1·0 c.c. is deducted for each 100 c.c. of medium. Then the calculated amount of alkali, less this deduction, is added, and a + 1·0 per cent. reaction is obtained. In other words, alkali is added insufficient to quite neutralize the medium (phenolphthalein neutral point), and the addition of 1·0 per cent. of $\frac{N}{0}$ alkali would still be required to make it completely neutral.

As an example of an actual standardization, the following is given:

Two separate 10 c.c. of a 1 litre of nutrient broth each required the addition of 1·3 c.c. $\frac{N}{10}$ sodium hydrate solution to make them neutral to phenolphthalein. Therefore, 980 c.c. (*i.e.*, 1,000 − 20 c.c.) will require $\left(\frac{1{\cdot}3}{10} \times 980\right) =$ 127·4 c.c. $\frac{N}{10}$ sodium hydrate solution. This is equal to 12·74 $\left(i.e., \frac{127{\cdot}4}{10}\right)$ c.c. of $\frac{N}{0}$ alkali.

One c.c. of $\frac{N}{0}$ acid is deducted for each 100 c.c., or 10 c.c. for the litre. Therefore, 2·74 (12·74 − 10) c.c. $\frac{N}{0}$ alkali is added to the medium, and a + 1·0 reaction is obtained.

If the medium is already alkaline to phenolphthalein, the sample removed for titration should be made acid by the addition of a definite and accurately pipetted amount of $\frac{N}{10}$ H_2SO_4. The mixture is then boiled and $\frac{N}{10}$ alkali run in in the ordinary way. A simple calculation gives the required result. This is preferable to the direct use of $\frac{N}{10}$ H_2SO_4 titrating to the neutral point.

Nutrient Broth.—That made from meat extract is recommended for ordinary purposes. It is slightly less nutritive than that made from raw beef, but is more uniform in quality, cheaper, and more readily made.

Weigh out 5 grammes Liebig's extract of beef (Lemco), 5 grammes of sodium chloride, and 10 grammes of Witte's

peptone, mix with 1 litre of distilled water in an enamelled saucepan, and boil until solution is complete. Transfer to a flask. Make up to 1 litre again, and steam in a sterilizer for three-quarters of an hour. Estimate the reaction, and bring to a + 1·0 reaction. Filter into a clean flask, and place in the steam sterilizer for half an hour. If not perfectly clear, filter again. Distribute into clean and preferably sterile test-tubes, about 10 c.c. for each tube, plug with cotton-wool, and sterilize in steam sterilizer for three successive days for half an hour each day.

Nutrient Fresh Beef Broth.— Many bacteriologists prefer broth made from fresh meat instead of extract of meat. To prepare, add 500 grammes of fresh beef, free from fat and finely minced, to 1 litre of distilled water. Place in an ice-chest or cold place for twenty-four hours. Strain through muslin, and make up to 1 litre with distilled water.

Add 10 grammes of Witte's peptone and 5 grammes of sodium chloride, and steam in steam sterilizer for one hour. The reaction is estimated, and the subsequent steps are the same as for Lemco broth.

Nutrient Gelatine.— Weigh out 5 grammes Lemco, 5 grammes of sodium chloride, and 10 grammes of Witte's peptone, and boil with a litre of distilled water in an enamelled saucepan. Transfer to a flask, and make up to 1 litre. Add 120 grammes of best 'gold label' gelatine (*i.e.*, 12 per cent.). Place the flask in the steam sterilizer, and steam for one hour to completely dissolve the gelatine. Estimate the reaction, and add the calculated amount of alkali to bring to a + 1·0 per cent. reaction.

As Eyre points out (Eyre, 1901), the increased bulk caused by the addition of the gelatine must be taken into account. According to this author, the final bulk, after the addition of 120 grammes of gelatine to 1 litre, measures 1,096 c.c., and the amount of $\frac{N}{0}$ alkali which has to be added must be estimated on this basis.

Cool down to about 50° C., and add the white of one egg. Mix well, and place in the steam sterilizer for rather over half an hour. The egg albumin is coagulated, and acts as a mechanical clarifying agent. The gelatine above the clots should be clear. Filter (preferably through *papier Chardin*) into a clean flask, funnel and flask being placed in the steam sterilizer. Put into tubes, 10 c.c. as nearly as possible into each, and sterilize for twenty minutes on three successive days.

Ten per cent. gelatine is usually recommended, but in hot weather this may have to be increased. Twelve per cent. is a satisfactory strength to use all the year round, and the advantage of always using the same strength gelatine is obvious. When made from fresh meat, the gelatine is added to the fresh meat broth instead of Lemco broth, as above.

Nutrient Agar.—Weigh out 5 grammes Lemco, 5 grammes of sodium chloride, and 10 grammes of Witte's peptone, and heat to boiling with a litre of distilled water in an enamelled saucepan. The solution must be distinctly alkaline. Transfer to a flask and make up to 1 litre.

Add 15 grammes (*i.e.*, 1·5 per cent.) of thread agar, broken up into small pieces. Digest in the autoclave at 115° C. for forty-five minutes. Estimate the reaction, and bring to a + 1 per cent. reaction. Cool to below 60° C., and add the white of an egg previously mixed with a little distilled water.

Heat in autoclave at 115° C. for forty-five minutes. Filter, preferably through *papier Chardin*, placing the whole apparatus in the steam sterilizer. Distribute into tubes, 10 c.c. into each tube, and sterilize, either once in the autoclave for thirty minutes at 115° C., or in the steam sterilizer for three successive days, twenty minutes each day.

The American Committee on Standard Methods recommends the addition of 2 instead of 1 per cent. peptone.

Litmus Milk.—Machine-separated milk can now almost always be obtained. It should be tested, to be certain that it is free from preservatives. Steam for half an hour in the steamer. Remove any coagulum from the surface. Estimate the reaction, and bring to a + 1 per cent. reaction. Add pure litmus solution to a suitable tint. Distribute into tubes, and sterilize in current steam for one hour on the first day and half an hour on the two following days.

Peptone-Water.—Make up a solution of 1 per cent. Witte's peptone and 0·5 per cent. sodium chloride in distilled water. Filter, tube, and sterilize in the autoclave.

Nitrate Broth.—Dissolve 10 grammes of peptone in 1 litre of ammonia-free distilled water, and add 2 grammes of nitrite-free potassium nitrate. Filter, tube, and sterilize for three days, thirty minutes each day, in the steam sterilizer.

Potato.—Select a number of fairly large potatoes. Wash well and scrub with a brush. Cut out cylindrical pieces

with a potato-borer. Cut each cylinder by a knife, if not already done by the borer, obliquely into two wedge-shaped pieces. Remove the ends of these, and soak them all night in water. Put into large test-tubes, each containing a piece of absorbent wool at the bottom. Add a small quantity of distilled water to each tube. Sterilize in the steamer for one hour on the first day and half an hour on the two following days.

Litmus Whey.—Fresh milk is gently warmed and clotted by means of rennet. The whey is strained off through muslin, and heated for one hour at 100° C. in the steam sterilizer. The solution is filtered, and pure litmus solution (azolitmin) is added to a convenient tint. The whey is then tubed, and sterilized in the steam sterilizer for half an hour on three successive days.

Sugar Media.—Durham's tubes (Fig. 9, p. 216) are the most convenient form of apparatus for testing the capability of different organisms to ferment the different sugars, alcohols, etc. They consist simply of a small test-tube, without a turned-over neck, placed in a larger one. The media in these tubes after three days' sterilization should completely fill the inner tube. Suitable media can be conveniently made as follows:

Liebig's extract of beef (Lemco) ...	5 grammes.
Peptone	10 „
Distilled water	1,000 c.c.

These are heated together as in making broth, transferred to a flask, made up to 1 litre, and steamed for forty-five minutes. The solution is made faintly alkaline to litmus-paper, and filtered into a clean flask. Five grammes of the appropriate sugar or alcohol (*e.g.*, lactose, glucose, mannite) are weighed out and dissolved in the warm solution. Pure litmus solution is added, to give a blue tint, and the mixture is tubed and sterilized for three days in current steam, twenty minutes each day.

To avoid inversion of the sugar, such media should never be heated above 100° C., and should be heated as little as possible.

Fresh-meat infusions should not be used in the preparation of sugar media, since they usually contain a certain amount of muscle sugar, etc. It is obviously imperative that these sugar media should contain only the added sugar

or alcohol, and no trace of other substances fermentable by the bacteria which are being studied. Care should be taken to ensure that the sugars and other substances to be fermented are obtained chemically pure.

Blood-serum.—A simple and convenient method of preparing this medium is the following :

Collect the blood at the nearest slaughter-house in a clean glass or enamelled metal cylinder. Cleanliness must be exercised, but it is not necessary that the blood be collected in a sterile condition. Remove to the laboratory, and place in the ice-chest for twenty-four hours. Pipette off the separated serum into a clean flask. Transfer to sterile test-tubes, about 5 to 6 c.c. in each tube. Place these in the steam sterilizer in a slanting position. Generate a moderate amount of steam, and leave the cover only loosely fitting, until the serum has quite solidified. This takes from one to three hours. Then fit the cover tightly, and subject to current steam at 100° C. for one hour. Sterilize again for the two following days at 100° C. for thirty minutes each day.

It is essential that the solidification shall take place below 100° C. If steam is generated in large amount, to the exclusion of all air, so that the medium is heated to 100° C. before complete solidification takes place, bubbles and cavities in the serum will be formed and the medium be spoilt.

Löffler's blood-serum mixture consists of 3 parts of blood-serum mixed with 1 part of 1 per cent. glucose broth. It is tubed, solidified, and sterilized as above. It is preferred by the writer to ordinary blood-serum, for most purposes.

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